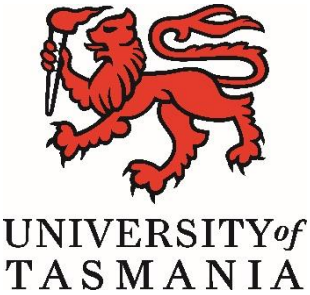

Colonisation Dynamics of *Listeria monocytogenes* Isolates from the Food Production Environment

by

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Abstract

Listeria monocytogenes is a ubiquitous saprophytic bacterium and human pathogen capable of causing severe disease in at risk population groups. The consumption of contaminated food products, particularly ready to eat products, is the main vector for human listeriosis. *L. monocytogenes* can enter the food production environment (FPE) through raw ingredients and can be further transmitted throughout the facility through staff movements leading to colonisation and persistence in the production environment. An understanding of the various elements which may contribute to *L. monocytogenes*' ability to colonise and survive within the FPE is therefore required to minimise the food safety risks of this pathogen.

This research assessed biofilm formation as it can be a key contributor to colonising the processing environment; the first aim was to develop a high throughput model biofilm system to assess biofilm formation at conditions reflective of FPEs, including low temperature and low nutrient conditions using common surface material present within processing facilities. This model system successfully enabled rapid screening of biofilm phenotypes, facilitating observation of attachment and biofilm formation. This development led into the second aim which centred on understanding a selection of colonisation dynamics utilising five fast biofilm formers and five slow biofilm formers identified using the model biofilm system. The strain set studied was referred to as the B10 group. No strong associations were identified between the growth rate, exopolymeric substance production and expression of signalling propeptide AgrD with the rapid colonisation phenotype. The global transcriptome suggested that transport, energy production and metabolism genes were widely upregulated during the initial colonisation stages under nutrient limited conditions. However, the upregulation of metabolic systems varied between isolates supporting the idea that *L. monocytogenes'* ability to colonise the FPE has strain-specific aspects.

The *L. monocytogenes* isolates (n=52) were phenotypically and genotypically assessed for their potential to survive within FPEs, as well as their pathogenicity and response to clinically relevant antibiotics. A vast array of genetic determinants was present across the collection with some strains containing important virulence genes suggestive of hypervirulence. As the strain collection was isolated from foods or the production environment all isolates contained multiple genes that aid tolerance and provide mitigation against a range of stressors. From data here a transposon identified in *Enterococcaceae* containing a novel *L. monocytogenes cadA* resistance gene was identified suggesting a horizontal gene transfer (HGT) event may have occurred between *Listeria* and *Enterococcus*. A novel insert was also identified in the hypervariable region in which some strains contain *Listeria* genomic island 1; this novel insertion shared similarity to Tn*916* from *Bacillus subtilis*. All isolates in this study were sensitive to the five clinically relevant antibiotics tested supporting the successful treatment of listeriosis by these antibiotics.

The adaptability of *L. monocytogenes* strains and the presence of antimicrobial resistance determinants opens the door to alternative treatment options within the FPE using biocontrol measures like bacteriophages, endolysins, competitive bacterial species, bacteriocins and plant-derived antimicrobial products. The applicability of bacteriophage has shown significant relevance against bacterial strains with the commercialisation of bacteriophage treatments; analysis of the literature showed bacteriocins and endolysins can offer significant reductions on established biofilms, however, they generally require further research and development. The use of competitive bacterial species can offer customised treatments when antagonist species are identified from the production environment. However, there have been minimal inhouse application of these biocontrol measures and as such further standardisation and in-facility assessment is required.

In summary, this thesis contributes to further our current understanding on *L. monocytogenes*' ability to colonise FPEs, and the survival, pathogenicity and treatment potential of strains

isolated from representative food environments were investigated. While the ability to colonise stainless-steel surfaces appeared to have some strain specific aspects it is noted that environmental conditions play a large part in this, and as such particular care in design and maintenance of the processing facility is required. In addition, strains isolated from the FPE environment display a vast phenotypic and genetic resistance profile with some isolates capable of hypervirulence. Regular monitoring of the phenotypic and genotypic profile is suggested with the identification of novel genes and inserts indicative of HGT events, which may contribute towards their enhanced fitness in the FPE and influence pathogenicity.

Declaration of originality

This thesis contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of my knowledge and belief no material previously published or written by another person except where due acknowledgement is made in the text of the thesis, nor does the thesis contain any material that infringes copyright.

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March 2021

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Publications and communications arising from this thesis

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Common abbreviations in thesis

BHI	Brain heart infusion broth
BHIA	Brian heart infusion agar
CFU	Colony forming units
DEGs	Differentially expressed genes
dBHI	Diluted brain heart infusion broth
DNA	Deoxyribonucleic Acid
EOs	Essential oils
FPE	Food production environment
h	Hour
min	Minute
phage	Bacteriophage
RNA	Ribonucleic acid
ST	Sequence type
SS	Stainless steel
µg	Microgram
µL	Microlitre
mL	Millilitre

Table of Contents

CHAPTER 1

Literature review and thesis objectives

Evolution of the foodborne pathogen *Listeria monocytogenes*.

Foodborne pathogens are a public health concern as they have the potential to cause food related illness and disease in humans. In Australia, food-borne illnesses account for an estimated 5.4 million cases per year and an estimated burden of $1.2 billion to personal and public health and the economy (1). Foodborne illnesses can be caused by a variety of viral, bacterial, parasitic or chemical agents; of these, the bacterium *Listeria monocytogenes* has emerged as a foodborne pathogen of public health importance (2). *Listeria monocytogenes* and non-typhoidal *Salmonella* are the main pathogens responsible for foodborne related deaths in Australia (3). During the 2001-2010 period, *L. monocytogenes* had a mortality rate between 20-30 % (1) in line with international rates (4-6). In addition, *L. monocytogenes* was frequently identified as the causative agent responsible for microbial food recalls (1).

Food recalls can have severe consequences on food businesses, with impacts affecting the brand name, the direct costs associated with the recall, loss of sales and the potential to also impact the wider commodity industry. Decreased consumer demand has been reported in the United States to last for 4-8 weeks following a food recall (7). In addition, Thomsen et al (8) reported economic effects may also impact other businesses marketing similar products to those implicated in foodborne outbreaks, despite not being related to the source of a specific contamination-associated outbreak, resulting in an industry wide issue. On a brighter note when food recalls are associated with products which have an established brand image, impacts can be limited to that brand. Product safety information provided by the offending company, industry or government informing consumers other products or brands are safe can reduce the length of the impact from a food recall as well as increase the sales of other products of the same commodity (8). Nevertheless, food recalls associated with the presence

of *L. monocytogenes* is a cause for concern requiring increased surveillance and understanding of how this bacterium is capable of colonising and surviving within the food production environment (FPE).

The genus *Listeria*

The genus *Listeria* consists of Gram-positive, rod shaped, non-spore forming facultative anaerobes and belongs to the phylum Firmicute, class Bacilli, order Bacillales and family Listeriaceae with only one other genus, *Brochothrix*. *Bacillus, Clostridium, Enterococcus, Streptococcus and Staphylococcus* phylogenetically are relatives of *Listeria* (9, 10). There are 23 currently published *Listeria* species (Table 1) (11-16), 15 of these species have been discovered since 2010 (10). The first *Listeria* species identified was *Listeria monocytogenes* in 1924 in a rabbit breeding program however it wasn't until 1940 in which the genus *Listeria* and *L. monocytogenes* name was settled upon after numerous variations (10, 17). Based upon the relatedness to *L. monocytogenes*, the genus *Listeria* can be divided into two groups: *Listeria sensu stricto* and *Listeria sensu lato* (Table 1) (11, 12). Schardt and colleagues (18) described the *Listeria sensu lato*, clade II, as being predominately associated with the natural environment and food associated surfaces, compared to the *Listeria sensu stricto*, clade I, as having been identified in the gastrointestinal tract, faeces and food products of animal origin indicating a relationship with mammalian hosts.

There are a variety of phenotypic tests that can be performed to identify *Listeria* isolates (Table 2). As a genus all *Listeria* species are catalase positive and oxidase negative, with further biochemical reactions representative of either the *Listeria sensu stricto* or *Listeria sensu lato* group. In addition, chromogenic agar, for example RAPID'L.MONO agar, can provide identification of *L. monocytogenes* within 24-48 hrs based upon the absence of xylose fermentation coupled with production of phosphatidylinositol phospholipase C (PIPLC) (19). Within the genus *Listeria*, only *L. monocytogenes* and *L. ivanovii* are considered pathogenic

with *L. monocytogenes* capable of causing disease in humans and animals, whereas *L. ivanovii* predominately infects ruminants, but rarely humans (20).

Table 1. Species within the genus *Listeria*

***Listeria sensu stricto* (Clade I)**	***Listeria sensu lato* (Clade II)**
L. monocytogenes	*L. grayi*
L. ivanovii	*L. fleischmannii*
L. innocua	*L. floridensis*
L. seeligeri	*L. aquatica*
L. welshimeri	*L. newyorkensis*
L. marthii	*L. cornellensis*
	L. rocourtiae
	L. murrayi
	L. weihenstephanensis
	L. grandensis
	L. riparia
	L. booriae
	L. dentrificans
	L. costaricensis
	L. goaensis
	L. thailandensis
	L. valentina

Table 2. Select phenotypic characteristics distinguishing the various *Listeria* species apart.

		Catalase	Oxidase	Motility	Growth at 4 C	Hemolysis	Voges Proskauer	Methyl red	Nitrate reduction	PI-PLC	Arylamidase	a-Mannosidase	D-Arabitol	D-Xylose	L-Rhamnose	a-Methyl-D-glycoside	D-Mannitol	Colony colour*	Halo*
***Listeria* sensu stricto**	*L. monocytogenes*	+	-	+	+	+	+	+	-	+	-	+	+	-	+	+	-	Blue	-
	L. marthii	+	-	+	+	-	+	+	-	-	-	+	+	-	-	+	-	White	-
	L. innocua	+	-	+	+	-	+	+	-	-	+	+	+	-	V	+	-	White	-
	L. welshimeri	+	-	+	+	-	+	+	-	-	V	+	+	+	V	+	-	White	+
	L. ivanovii	+	-	+	+	+	+	+	-	+	V	-	+	+	-	+	-	Blue	+
	L. seeligeri	+	-	+	+	+	+	+	-	-	+	-	+	+	-	+	-	Yellow	-
Listeria* sensu lato*	*L. aquatica*	+	-	-	-	-	V	+	+	-	-	+	-	+	+	-	-	White	-
	L. booriae	+	-	-	+	-	-	+	+	-	-	+	+	+	+	+	+	White	-
	L. cornellensis	+	-	-	+	-	-	V	+	-	-	-	-	+	-	+	-	White	-
	L. costaricensis	-	-	+	-	-	+	ND	+	-	-	-	+	+	+	+	-	ND	
	L. fleischmannii	+	-	-	-	-	-	+	+	-	-	-	+	+	+	+	V	White	-
	L. floridensis	+	-	-	-	-	-	+	-	-	-	-	-	+	+	+	-	White	-
	L. goaensis	+	-	-	-	+[α]	-	+	-	-	-	-	+	+	+	-	-	ND	
	L. grandensis	+	-	-	+	-	-	+	+	-	-	-	V	+	-	+	-	White	-
	L. grayi	+	-	+	+	-	+	+	V	-	+	V	+	-	-	+	+	White	-
	L. newyorkensis	+	-	-	+	-	-	+	+	-	-	-	-	+	V	+	+	White	-
	L. riparia	+	-	-	+	-	-	V	+	-	-	+	-	+	+	+	V	White	-
	L. rocourtiae	+	-	-	+	-	-	V	+	-	-	+	-	+	+	+	+	White	-

L. thailandensis	+	-	-	-	-	+	ND	+	-	-	-	V	+	+	+	-	ND	
L. valentina	+	-	-	-	-	-	ND	-	-	-	-	++	+	+	-	-	ND	
L. weihenstephanensis	+	-	-	+	-	-	V	+	-	-	-	+	+	+	+	+	White	-

Adapted from Allerberger et al (19); Orsi and Wiedmann (12); Weller et al (21); Gasanov et al (22); Quereda et al (15); Leclercq et al (16); Doijad et al (13); Núñunez-Montero et al (14); and Bio-Rad Laboratories Inc. (23). * RAPID'L.mono chromogenic plates phenotype, α alpha haemolysis. ** *L. murrayi* (heterotypic synonym of *L. grayi*) and *L. dentrificans* (transferred to genus *Jonesia* as *J. dentrificans*) is excluded from the list. PI-PLC phosphoinositide phospholipase C, + positive, − negative, V variable,

Listeria monocytogenes

L. monocytogenes cells are approx. 0.4-0.5 μm in diameter and 0.5-2 μm in length, are capable of growing between pH 4.2 to 9.5 and at temperatures ranging from -0.4 to 50° C, with the production of peritrichous flagella resulting in a tumbling motility most active between 20 and 25 °C (24-26). Phylogenetically, *L. monocytogenes* has four distinct lineages (I, II, III and IV), with the majority of isolated strains associated to lineage I and lineage II (27). Initial studies reported lineage specific associations; for example, human listeriosis cases are predominately from lineage I and are considered more pathogenic compared to lineage II isolates, which are reportedly isolated more frequently from food and food-related environments. Lineage III and IV generally cause disease in animals (27-29), however international and national regional differences ultimately account for variances in reported associations, particularly regarding lineage I and II. Across the lineages, *L. monocytogenes* isolates can be divided into 13 different serotypes (Table 3). Serotyping of *Listeria*, first developed by Paterson (1940) and further advanced by Donker-Voet (1957) and Seeliger and Höhne (1979) (27), determines differences based upon the identification of somatic O-antigens and flagella H-antigens in a slide agglutination test using standardised high quality antisera (30-32). Amongst the *Listeria* species somatic antigens there have been 15 O-antigen subtypes (I-XV) and four H-antigen subtypes (A-D) classified; a combination of O and H antigens establishes the serotype (31). Slide agglutination serotyping has provided the historical subtype classification that is known today; however, this process is time consuming, requires expensive high-quality antisera, and is not conducive to analysis of a large number of isolates. It is also unable to designate *L. monocytogenes* strains in blind *Listeria* sample analysis, since multiple *Listeria* species may possess the same serotypes (30-32).

Table 3. *Listeria monocytogenes* genetic lineages and serotypes

Lineage	Serotype	Association
I	1/2b, 3b, 4ab, 4d, 4b, 4e	Human listeriosis outbreaks
II	1/2a, 1/2c, 3a, 3c	Animal and human listeriosis cases and food-related sources
III	4a, 4c, 4b	Rare, predominately animal origin
IV	4a, 4b, 4c	Rare, predominately animal origin

Adapted from Valderrama and Cutter (7), Orsi et al (27) and Camargo et al (28).

Many methods to subtype *L. monocytogenes* have been developed based upon phenotypic, biochemical and genetic differences (Table 4). While a majority of the newly developed phenotypic and biochemical methods were improvements on their predecessors, they were still inefficient methods due to the time required, lack of cost effectiveness, lack of sensitivity, specificity and reproducibility (30, 32-34). Advances in technology saw a move from phenotypic to genetic analysis using molecular techniques with the use of restriction digestions or deoxyribonucleic acid (DNA) amplification. Restriction digestions rely on specific enzymes to digest DNA into smaller fragments; two common techniques include ribotyping and pulsed field gel electrophoresis (PFGE). PFGE has been dubbed the gold standard of subtyping *Listeria* for many years (30, 32, 35, 36). It predominately uses *Asc*I and *Apa*I restriction enzymes to digest genomic DNA into typically 8-25 larger fragments of around 40 to 600 kilobases, which are then electrophoresed on an agarose gel. The DNA banding patterns are compared and allocated into different pulsotypes (30, 31). Although PFGE is a time consuming and labour intensive method that requires skilled personnel, expensive restriction enzymes, specialised equipment and initially created concerns associated with interlaboratory reproducibility, it remained a mainstay as a preferred method since it displayed high discriminatory power, superior to many alternative approaches (30, 37). The reproducibility was improved with the inclusion of *Listeria* into PulseNet USA, which is a national United States surveillance network to detect foodborne disease outbreaks through the comparison of

DNA fingerprints of pathogenic bacteria (*Salmonella, Shiga toxin-producing Escherichia coli, Campylobacter, Yersinia, Shigella* and *Listeria*) by local, state and federal health laboratories (38-42).

In 2001, a standardised operating procedure was developed for PFGE subtyping of *Listeria* reducing the multiday protocol to 30 hours allowing for real-time analysis of listeriosis outbreaks (43). The success of PulseNet USA is estimated to have reduced foodborne illness by 270,000 cases per year, with a significant decrease in medical and loss of productivity costs in comparison to the actual cost to run the surveillance program (41, 42). As such, PulseNet International has evolved incorporating 86 countries and allows the sharing of information to assist in detecting international outbreaks (35, 41).

Although PFGE typing has been considered the gold standard for source tracking, it was limited as a tool to understand population genetics across the species. Thus, the development of multi-locus sequence typing (MLST) brought about an alternative subtyping method, which offered greater insights into evolutionary patterns across the species. A variety of MLST methods have been developed which utilises either housekeeping, stress or virulence related genes or a selection from each (54). The MLST scheme developed by Ragon et al (55) has been the most common method utilised, includes sequence analysis of seven housekeeping genes, and initially application was through PCR amplification and subsequent sequencing (Table 5). As whole genome sequencing has become increasingly cheaper, there has been a shift towards genomic subtyping utilising associated datasets. The housekeeping genes can be extracted from the genome sequence data, and MLST type determined, resulting in inter-method compatibility (56). The subsequent development of core genome MLST has resulted in increased discrimination between strains, rapid strain characterisation, an understanding of evolutionary relationships amongst *L. monocytogenes* strains and improved epidemiological investigations and source tracking (30, 55, 56).

Table 4. Techniques used for subtyping *Listeria monocytogenes* isolates.

Technique	Methodology	Reference
Serotyping – antisera	Somatic and flagella antigens by slide agglutination	(44)
Serotyping - PCR	PCR primers determined from previously serotyped strains	(45)
Phage typing	Susceptibility or resistance to Listeria specific bacteriophages	(46)
Multi-locus enzyme electrophoresis (MLEE)	Differential electrophoretic mobility of bacterial enzymes	(33)
Ribotyping	Chromosomal DNA digestion by frequently cutting restriction enzymes	(33)
Pulse field gel electrophoresis (PFGE)	Chromosomal DNA digestion by rarely cutting restriction enzymes	(43)
Amplified fragment length polymorphism (AFLP)	Selective amplification of genomic restriction fragments by two enzymes and site-specific ligation	(47)
Random amplified polymorphic DNA (RAPD)	Singular primer PCR of a random sequence, degree of homology determines typing profile	(48)
Repetitive extragenic palindromic and enterobacterial repetitive intergenic consensus (REP and ERIC PCR)	Repetitive element sequence-based PCR	(49)
Multilocus sequence typing (MLST)	Single nucleotide polymorphism of 7 housekeeping genes from dispersed genomic locations	(50, 51)
Multi virulence locus sequence typing (MVLST)	Single nucleotide polymorphism of 6 virulence and virulence associated genes	(52)
Multilocus variable number of tandem repeats analysis (MLVA)	Variances of the copy numbers of tandem repeats at specific loci	(53)
Whole genome sequencing (WGS) core genome MLST (cgMLST)	Extension of MLST, gene by gene allelic profiling utilising > ~1700 core genome loci to determine cgMLST types.	(56)

Table 5. The 7 housekeeping genes used in the Ragon et al. scheme to determine MLST of *L. monocytogenes*.

Locus	Gene function
abcZ	ABC transporter
bglA	Beta glucosidase
cat	Catalyse
dapE	Succinyl diaminopimelate desuccinylase
dat	D-amino acid aminotransferase
ldh	L-lactate dehydrogenase
lhkA	Histidine kinase

Subtyping *L. monocytogenes* strains allows for genetic characterisation to understand the evolution and ecology of *L. monocytogenes*, virulence potential, the associated risk to public health, along with the development of epidemiological markers (28, 57, 58). These markers allow for the identification of sporadic listeriosis cases and outbreaks, identification of the genotype of strain responsible, and thus aid in source tracking investigations to determine which cases are linked, the food item implicated and/or the facility associated, thus allowing mitigation procedures to be implemented (28, 57, 59, 60). In addition, environmental adaptations that have occurred will be observed; for example, the development of resistance to disinfectants, heavy metals, and antibiotics, as well as identification of geographical-associated differences (61, 62). Although there are many subtyping methods available which provide greater discriminatory ability between isolates, serotyping is still used as a reference point in many publications due to the amount of historical data serotyping initially provided.

Listeriosis

Listeriosis is a food borne disease, typically associated with the ingestion of food products contaminated with *L. monocytogenes*. It can manifest as a non-invasive febrile form of gastroenteritis or if it crosses the epithelial barrier, as invasive listeriosis resulting in

septicaemia, meningitis or meningoencephalitis (6, 63-65). Population groups most at risk of developing invasive listeriosis include the elderly, immunocompromised, pregnant women and neonates (4, 6, 63, 66). Pregnant women may present with non-invasive listeriosis or be asymptomatic, with transmission to the foetus or neonate occurring *in utero* or during the birthing process, potentially resulting in abortion, still birth, preterm birth and septicaemia or meningitis following birth (63, 66, 67). In comparison to healthy individuals', pregnant women are at greater risk of infection, with increased risk also associated to particular ethnicities, for example Hispanics, as a result of cultural dietary differences (68) with approximately a third of infections in expectant mothers resulting in preterm birth, still births or miscarriages (69). In non-pregnancy related invasive listeriosis cases, it can take up to 70 days before symptoms present, and this long incubation period can make it difficult in identifying the associated contaminated food product responsible (24).

Many occurrences of listeriosis are sporadic; however, occasionally national or multinational outbreaks occur. Notable examples include the large 2017-2019 outbreak in South Africa that resulted in more than 200 deaths and over 1000 laboratory-confirmed cases. Although the outbreak only occurred in South Africa, other Sub-Saharan African countries were also sold the contaminated meat product (70, 71) that was the source of the outbreak. In 1998 and 1999 a multistate outbreak across the United States resulted in 101 cases and 21 deaths (72). A multi-province outbreak in Canada during 2008 saw 24 fatalities and 57 human infections (73). During 2014-2015, a multistate outbreak in the United States resulted in 35 cases in total with 34 requiring hospitalisation and that ended with seven fatalities (74). In 2018, a multistate listeriosis outbreak was identified in Australia resulting in 22 cases, one miscarriage, seven deaths and two cases genetically linked to an outbreak identified in Singapore as the product responsible was also distributed internationally to eight countries (75, 76).

Septicaemia, meningitis and meningoencephalitis are forms of disease that can arise from invasive listeriosis and require antibiotic treatment. Most *L. monocytogenes* strains are

susceptible to a wide range of antibiotics; however, an innate resistance to fosfomycin, cefotaxime, cefepime, oxacillin and lincosamides exists (77). The preferred antibiotic treatment is with a β-lactam (penicillin, ampicillin or amoxicillin) singularly or in combination with an aminoglycoside like gentamicin (77, 78). β-lactams alone provide a bacteriostatic effect and have been proven to be effective in the treatment of listeriosis, however for patients over 50 years with a pre-existing disease, transplant or respiratory support, it is suggested gentamicin is used in combination, providing a bactericidal effect (78). For patients with β-lactam allergies, trimethoprim in combination with sulfamethoxazole is available. Various other antibiotics have also shown to be effective including vancomycin, erythromycin, tetracycline, rifampicin and fluoroquinolones (77, 78). Either way the antibiotic of choice needs to be able to permeate the host cell with minimal to no change in concentration or pH to remain effective (79). Importantly, antimicrobial resistance in *L. monocytogenes* isolates is beginning to be reported more frequently, with numerous studies performing surveillance on antibiotic sensitivity against a range of antibiotic classes and reporting increasingly higher incidence of single or multiple drug resistance (80-86). Increased resistance to antibiotics within *Listeria* species has been linked to improper use in agriculture, animal production and human medicine, with variances in antibiotic resistance the result of regional variance in antibiotic misuse (84, 86, 87). The increased presence of antibiotics within the environment and/or clinical settings can create selection pressures which can trigger the development and horizontal transfer of resistance determinants (86), particularly in niche environments like biofilms. In addition, the identification of strains displaying co-selection with a disinfectant and antibiotic or heavy metal have resulted in increased tolerance to the involved antimicrobials, along with other antibiotic classes (88).

Pathogenicity

After ingestion of contaminated food products, *Listeria* which survive the gastric environment colonise gastrointestinal cells by binding to surface receptors and intentionally induce

endocytosis in endothelial cells and M cells of Peyer's patches, to translocate through the intestinal membrane where it subsequently translocates to various tissues (89-91) (Figure 1). *Listeria* is engulfed into a vacuole by professional phagocytic cells including macrophages as a host defensive mechanism (89-91). In response, a listerial haemolysin called listeriolysin O and other factors are secreted to degrade the vacuole; *Listeria* then enters the cytosol (89-92). Multiplication and cell to cell spread is due to the action of ActA, the actin assembly-inducing protein, which initialises and polymerises host cells actin fibres to form actin tails moving *Listeria* towards the host cell membrane (89-91). The pseudopod like protruding structures assist in phagocytosis by neighbouring cells and the resultant intracellular invasion (89-91). Escape from the double membrane vacuole utilising the same gene products involved in the primary vacuole breakdown signals the completion of an infectious cycle and the process repeats (91, 92).

Listeria monocytogenes pathogenicity is set apart from other *Listeria* species as the result of pathogenicity islands and in particular, a 9-kb chromosomal island termed *Listeria* pathogenicity island 1 (LIPI-1), also known as the *prfA* virulence cluster. This genetic loci contains six genes responsible for important steps throughout the intracellular lifecycle (20, 93). The LIPI-1 island is bordered by *prs*, a putative phosphoribosyl pyrophosphate synthetase specific to the genus *Listeria*, and the *orfX* gene of unknown function located up stream of *ldh*, lactate dehydrogenase (20, 45, 93). LIPI-1 contains the transcriptional activator *prfA* which transcribes 140 genes either directly or indirectly and has been termed the master regulator. Adjacent to *prfA* in LIPI-1 is *plcA* and *hly* that code phosphatidylinositol-specific phospholipase C (PI-PLC) and listeriolysin O (LLO). These proteins enable escape from vacuoles. The next genes on LIPI-1 include *mpl*, zinc-metalloenzyme; *actA*, actin inducing assembly protein; and *plcB*, a broad range phospholipase C (PC-PLC) all responsible for cell to cell spread and actin-based motility (20, 93-96). There are two surface proteins, internalins A and B (*inlA* and *inlB*), also important in virulence, crucial for entry into non-phagocytic cells, which are located outside the LIPI-1 island, comprising the *inlAB* islet (96, 97).

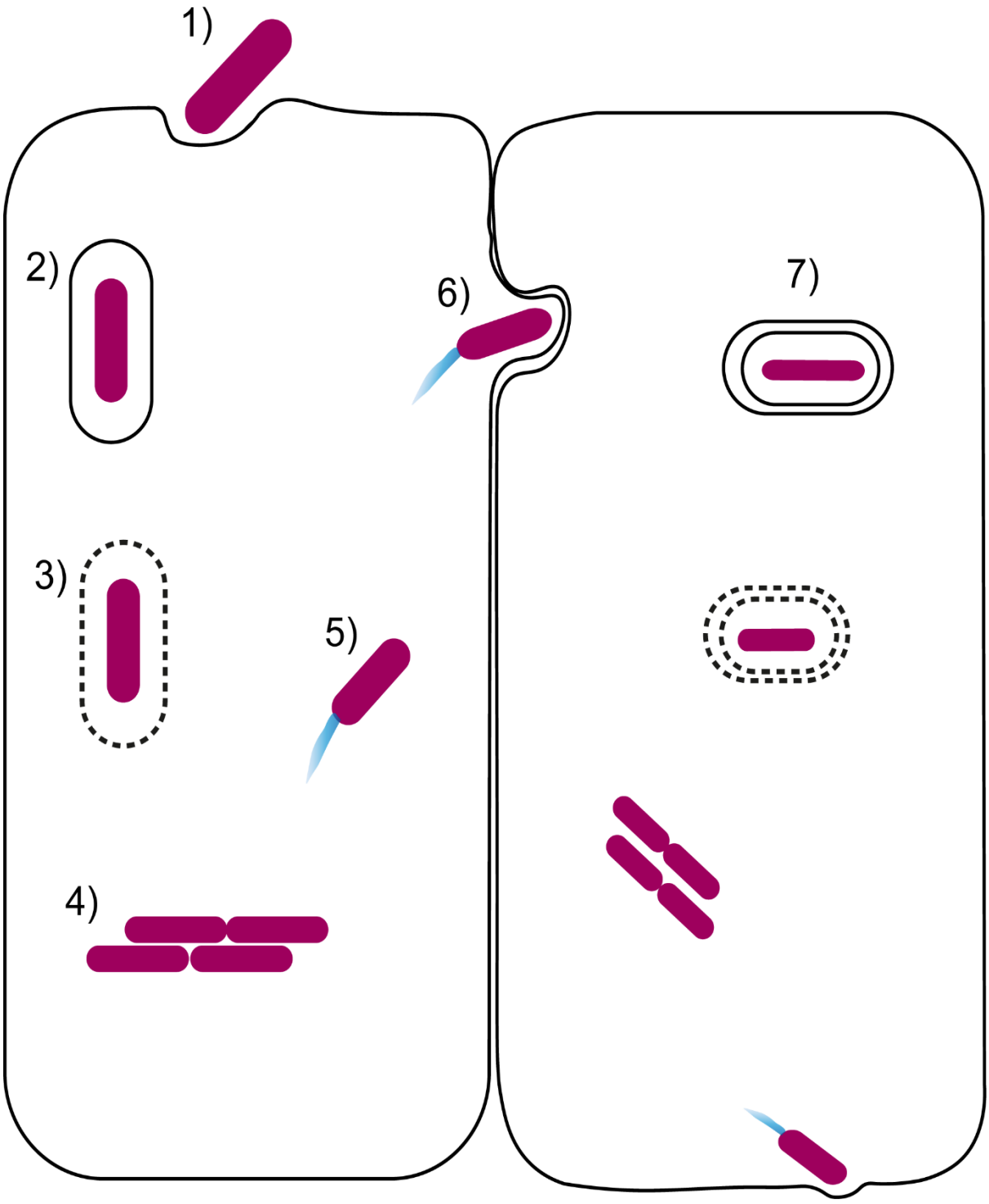

Figure 1. The intracellular lifecycle of *Listeria monocytogenes*. The *Listeria monocytogenes* intracellular lifecycle occurs through several broad steps: 1) *L. monocytogenes* enters cells resulting in the 2) formation of vacuoles, 3) vacuole lysis allows *L. monocytogenes* to escape 4) where cells are able to replicate, 5) actin-based motility occurs, 6) allowing cell-to-cell spread, 7) the intracellular lifecycle begins again. Adapted from Luque-Sastre et al (11), Bhunia, A.K. (98), Welch, M.D. (99), and Kühbacher et al (100).

The InlA protein is of particular interest as mutations in this protein have been identified resulting in a truncated and non-secreted form of InlA, a result of premature stop codons (PMSCs) and polymorphisms in the associated coding gene (101, 102). To date, 21 *inlA* mutation types have been identified (97, 103, 104) with the majority of *L. monocytogenes* isolates with InlA truncations associated with food sources (101). Strains with InlA truncations have resulted in attenuated virulence and invasion in guinea pig models and Caco-2 intestinal epithelial cells (105). Nonetheless, *L. monocytogenes* strains with *inlA* PMSCs have been identified in human listeriosis cases suggesting these isolates still have the potential to cause disease. Although the full repertoire of additional factors at play likely remain unresolved, additional surveillance of *inlA* mutations is required. While these are only a selection of some key virulence factors the *L. monocytogenes* genome contains, there are many other genes involved in host survival and various stages of disease development.

Ecology

As a ubiquitous environmental bacterium, *L. monocytogenes* can inhabit various niches from life within eukaryote hosts to natural and urban environments (Figure 2). As a saprophyte, the natural reservoir of *L. monocytogenes* is decaying vegetation (106); however due to its ability to adapt to a vast range of conditions and environments, it has also been isolated from soils, farms, surface water and in urban environments such as waste water and raw foods like fruits and vegetables in addition to domestic and wild animals (107-111).

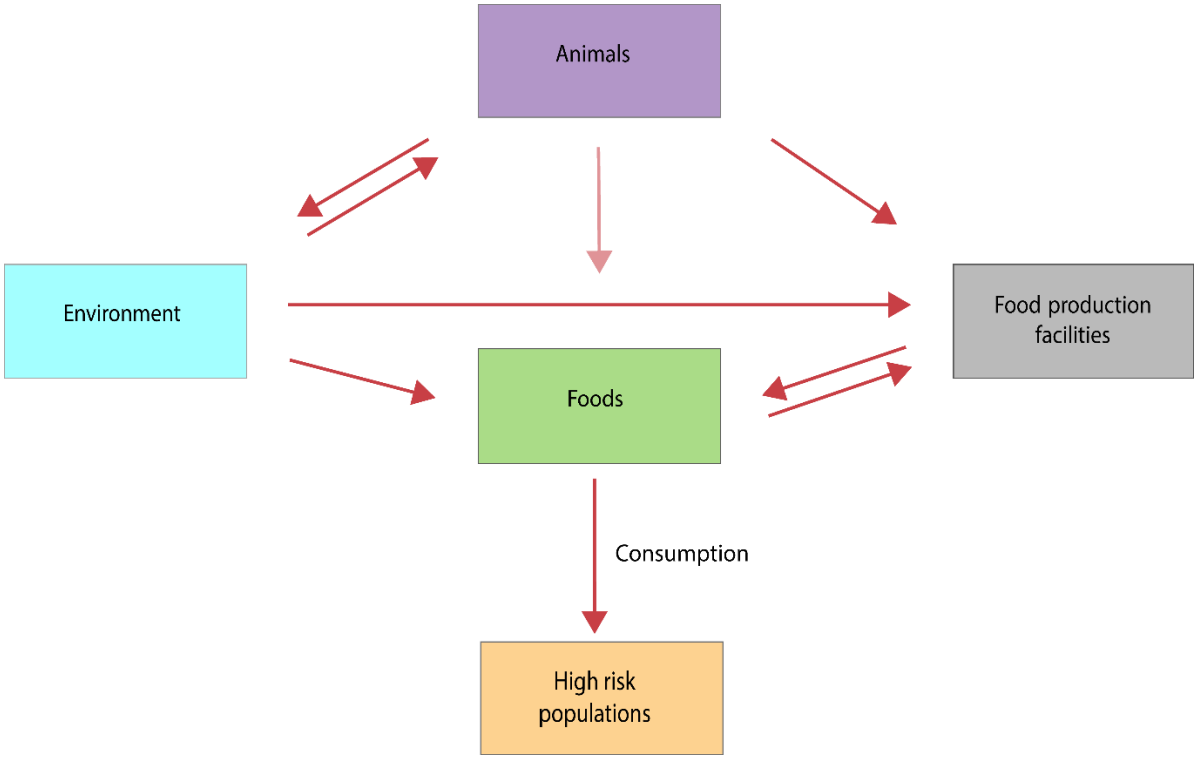

Figure 2. The farm to fork transmission cycle of *Listeria monocytogenes*. Adapted from NicAogáin and O'Bryne (116) and McMullen and Freitag (117).

Listeria in farm environments

The farm environment is a common isolation source of *L. monocytogenes*, whether it is a herd animal or fresh produce-associated farms. Infections in farm animals can result in similar disease as invasive listeriosis in humans; primarily encephalitis, bacteraemia and septicaemia, which can also result in transfer of the infection to the foetus and possible abortion (112). In addition, infections of the mammary glands have also been reported in goats, cows and sheep (113, 114). The poor fermentation and subsequent storage of silage, particularly during the colder months, has been reported as a common source of contamination, along with food and water troughs, bedding and other surfaces in close contact to farm animals (113). There are numerous factors influencing *L. monocytogenes* presence within the farm environment, with strong links tied to the use of silage feeding and increased faecal shedding by farm animals (106). The hypothesis for *L. monocytogenes* transmission

throughout the farm environment involves initial contamination of crops, soil and silage may occur from wildlife, birds and rodents, the contaminated soil may then be used as a fertilisation source leading to exposure and infection of animals during grazing or when they consume contaminate silage (106, 107). Ruminant farm animals, in particular, can be asymptomatic or carriers of *L. monocytogenes*, and contaminate the farm environment, crops, shelter, and processing environment and equipment through faecal shedding (107, 114, 115). In a study by Schoder and colleagues (106), *L. monocytogenes* was present on employee work boots two times more than silage, and transmission from the dairy farm to the milk processing environment was predominately related to free movement of farmers between the two environments.

Contamination of the farm environment is not limited to livestock production, and recent outbreaks have seen increasing frequency in other vehicles for contamination, such as fruits and vegetables including cantaloupes, apples, celery, stone fruit and salad ingredients like green leafy produce, with contamination linked to the preharvest environment in some cases (118-120). Irrigation water, soil fertilised with untreated manure, and water runoff from nearby livestock paddocks, are some of the reported sources of preharvest contamination (120). Unlike livestock farms, horticulture and grain production are not exposed to frequent shedding events with the exposure risk predominately the result of contaminated irrigated water, with increased isolation of *L. monocytogenes* within the first 24 hours of either irrigation or precipitation (121).

Although *L. monocytogenes* can be isolated from various environmental water samples, soils and sediments, the likelihood of *L. monocytogenes* presence in fish farms seems to be less prevalent than livestock or dairy farms. Sediments and sea bottom soil have been shown to preserve *L. monocytogenes* for long periods of time and can become contaminated with water runoff from rivers, brooks or other water sources during rain events, the type of fish pond or channel utilised (and the various hygiene practices utilised or not utilised with the various

ponds) if the water source is used for aquaculture purposes or alternatively the water source may naturally contain *L. monocytogenes* which may be associated with areas that have a high organic load (122-124).

Listeria monocytogenes and the food production environment

The introduction of *L. monocytogenes* isolates into the processing environment can be the result of raw ingredients (106, 113, 120, 124) and result in contamination of food products. Even incidental contamination from food or raw ingredients, the external environment or movement of facility workers can result in the introduction of *L. monocytogenes* to the FPE. However, additional research is still required to elucidate the true presence and transmission of *L. monocytogenes* across various supply chains of food processing industries.

Persistence

Due to their ubiquitous nature, *L. monocytogenes* is a difficult microorganism to prevent from entering and colonising food production facilities, with some strains being identified as persistent. Persistent strains act as a repetitive source of cross contamination, with reports of repeated isolation of persistent strains from food processing facilities over numerous years. For example, in a chilled food processing plant a strain was found to persist over an eight year period (125). Similarly, a single pulsotype dominated over a seven year study at an ice-cream facility (126). Strain persistence in a cold smoked salmon plant over four years (127) and frequent isolation of another persistent genotype over three years in a pig slaughter and processing plant have been reported (128). Although no formal criteria defining persistence exists, the consensus is isolates that are indistinguishable from one another need to be repeatedly identified during surveillance of the FPEs over a sufficient period of time (129-131). However, the molecular methods used, number of samples isolated, and the sampling duration is objective and therefore inter-study variations occur. Due to this, Stasiewicz *et al* (131) considers the concept of persistence to be more of an empirical set of rules which may

miss-report true persistence problems. For example, frequently reintroduced strains may fit these rules and be recorded as a persistent strain when in fact the FPE has poor hygiene barriers preventing reintroduction or cross contamination (131).

Most studies (128, 132-136) identify serotype 1/2a as frequently associated with persistent strains identified in FPEs, which is supported by the association of this serotype with food sources. Serotypes 4b, 1/2b and 1/2c occurrence is generally lower in FPEs, however, persistent strains of these serotypes have been reported (7, 27). The increased genomic analysis of food and production environment strains has resulted in the identification of *L. monocytogenes* MLST sequence types which can be over-isolated in these categories. Sequence type 121 and 9 are described as food associated clones whereas isolates from CC1, CC2, CC4 and CC6 are predominately associated with clinal isolates and infections relating to the central nervous system or maternal-neonatal listeriosis (137, 138). ST121 has been identified across a variety of different food matrices with Felix et al (139) suggesting ST121 strains have an increased colonisation capability across a larger diversity of food categories. Whereas ST9 isolates have been associated with meat products and production facilities as it has been implied, they are better adapted to the associated conditions of this category (140).

How persistence occurs is also an area of debate between researchers, with one model suggesting specific phenotypic and/or genotypic traits to be responsible for a strains ability to persist (4, 141, 142). Other studies associate persistence as a random occurrence based upon a strain finding an appropriate niche at the right time (143, 144). Studies have looked at stress response differences between persistent and non-persistent or sporadic strains, with varying results. For example, Magalhaes et al (145) found persistent strains responded better to variances in temperature, acidic and high salt conditions in comparison to non-persistent strains, however no differences were noted in response to sanitisers. Lundén et al study (146) only observed differences in strains in varying acidic conditions. Whereas another study

reported persistent strains were more resistant to disinfectants than non-persistent strains (147).

Irrespective of the strain type, the ability to persist is of hygienic and economic importance to food producers and managers. Factors influencing the persistence and survival of *L. monocytogenes* in food production facilities are influenced by a number of genetic and environmental elements. These include the bacterium's ability to adhere to surfaces and form biofilms, survive in varying temperatures and acidic environments, along with the unhygienic design of the facility or equipment, worn or damaged equipment or surfaces and hard to reach places which can create niches that *L. monocytogenes* can proliferate in and/or form biofilms, negatively impacting effective cleaning and sanitising procedures (7, 24, 129, 143, 148-151).

Biofilms

Biofilms are structures containing microbial cells attached to each other and/or a surface housed in an extracellular polymeric substance (EPS) (142, 152) that offers:

- increased protection from the environment, for example from cleaners, disinfectants and desiccation,
- removal of toxic metabolites,
- transfer of nutrients between biofilm residents and,
- the opportunity to acquire new genetic traits, like antibiotic or potentially disinfectant resistance genes through horizontal gene transfer (129, 153).

In addition, bacterial cells within biofilms can be in various metabolic states, further compounding the protective conditions afforded from environmental stresses.

Biofilm formation consists of five stages of development (Figure 3) starting with planktonic bacteria located on either a biotic or abiotic surface where initial attachment occurs through the use of van der Waals forces, hydrophobic interaction or electrostatic forces (2, 154).

Irreversible attachment follows, resulting in the production of the EPS and aggregate formation where cells begin transitioning out of the free-swimming state characteristic of planktonic bacteria (154, 155). The biofilm develops during the first stage of maturation where cell numbers increase and are differentiated into specific community roles, and the EPS begins to form structures to sustain the biofilm community (154-156). The biofilm hits peak maturation when the EPS is at maximum thickness and the presence of complex three-dimensional structures for the dispersal of nutrients and waste is complete (2, 154, 155). The final stage of biofilm formation is dispersal, where cells become dislodged from the colony to spread and colonise new surfaces and undertake the biofilm lifecycle again (2, 154).

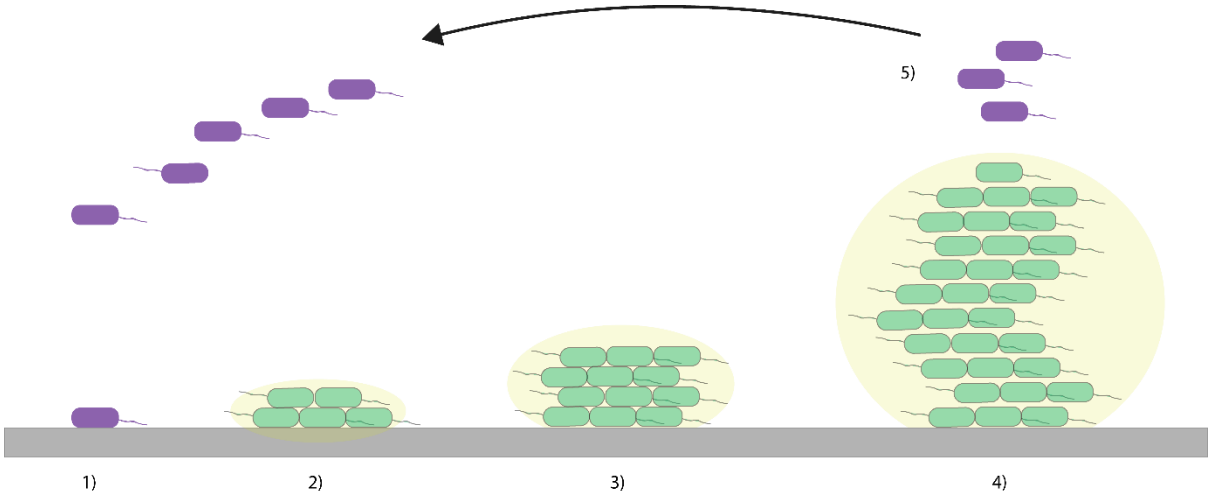

Figure 3. The biofilm formation cycle. The five stages of biofilm formation involves 1) initial attachment by planktonic bacteria, 2) irreversible attachment along with the production of EPS, 3) the first stage of maturation where the biofilm grows, 4) the second stage of maturation where the biofilm reaches peak growth and 5) dispersion where biofilm cells detach to continue the cycle. Adapted from el Moustaid et al (154), da Silva et al (2) and Vasudevan (161).

Biofilm formation by L. monocytogenes

Initial biofilm research in *L. monocytogenes* focused on determining biofilm formation ability between lineage, serotype or persistent strains, with some studies reporting high biofilm

production was associated with lineage I strains (144), lineage II strains (142), serotype 1/2a (157), persistent isolation (142), surface type (158) or increasing temperature (159); whereas other studies were reporting no association with a chosen subtype (160), origin of isolation (159) or persistence (144, 157). Interesting observations have been reported surrounding the availability of nutrients during biofilm formation, with a study by Kadam et al (159) reporting enhanced biofilm formation occurring in the early stages of limited nutrient conditions, suggesting times of limited nutrient availability within the FPE may stimulate the biofilm lifecycle.

While many researchers have looked at *L. monocytogenes* ability to form biofilms at clinically relevant conditions, there is less research using conditions reflective of the FPE and within this, there is conjecture between the relationship between serotype, lineage, persistence and the ability to form biofilms (142). More recent studies have indicated biofilm formation is strain-specific, with environmental and niche specific conditions significant contributors (143, 159, 162, 163). Unlike *Pseudomonas* species which are very prolific biofilm formers, *L. monocytogenes* can be classified as an intermediate producer, as the extent of biofilm formation can be influenced greatly by environmental conditions and surface type (164). It has been reported *L. monocytogenes* biofilms can reach up to 4-6 log CFU/cm^2 which is 3-6 log CFU/cm^2 lower than other bacterial species (2, 164). In addition, the mushroom-like structure characteristic of *Pseudomonas* biofilms is not present in *L. monocytogenes* biofilms (156, 165). Instead the architecture of *L. monocytogenes* biofilms varies depending on conditions utilised. Under optimal conditions and nutrients *L. monocytogenes* displays a characteristic honeycomb-like structure with hollow voids consisting of dead cells and extracellular DNA (eDNA). Various authors have reported the honeycomb-like structure indicative of a complex and mature biofilm (155, 164, 166, 167). Guilbaud et al (167) also reported the presence of flagella in biofilms producing the honeycomb-like structure, whereas a lack of flagella resulted in flat unstructured biofilms. The honeycomb-like structure is reported to offer improved structural stability and nutritional absorption and therefore a fitness advantage (164). The

presence of dead cells in the hollows provides nutrients for surviving cells while releasing eDNA, which also influences structural stability along with spatial organisation of the biofilm (167). However, for the most part *L. monocytogenes* cells within the FPE do not have access to consistent nutrients and therefore Cherifi et al (168) compared biofilm structure between a rich nutrient medium and a low nutrient medium. In full nutrients *L. monocytogenes* was capable of forming multilayer biofilms. In comparison, under nutrient limited conditions a knitted network of elongated cells different to the honeycomb-like structures reported in other studies was present, along with increased presence of dead cell biovolume suggesting low nutrient conditions enhance cell death and provides a structural benefit through the release of eDNA (168).

Within the FPE biofilms typically consist of multiple species, with the microflora varying depending on the origin of isolation. Multispecies biofilms can display a cooperative interaction among biofilm residents where increased adhesion, growth or biofilm formation may occur along with the sharing of nutrients and increased protection from disinfectants (169) or alternatively a competitive interaction may result in one species dominating or excluding another species through the consumption of nutrients or the production of antimicrobial compounds (169). Microflora analysis with a focus on floor drains as areas of importance can elude to *Listeria*-positive and *Listeria*-negative niches. An analysis of a meat processing facility by Fox et al (170) identified *Listeria*-positive and *Listeria*-negative drains comprised of different proportions of *Lachnospiraceae*, *Pseudomonadaceae* and *Rikenellaceae* as the most abundant families within the two drain types; however, only three families were found to reside exclusively in *Listeria*-negative drains compared to 21 families within the *Listeria*-positive drains. The importance of *Listeria*-negative niches was further examined by Zhao et al (171) which identified nine isolates displaying inhibitory actions to a five-strain mixture of *L. monocytogenes*, with two of these isolates ultimately being highly inhibitory to *L. monocytogenes* biofilms. Further assessment in a poultry processing facility through an enzyme-foam-based cleaning agent with the two strains of interest added, resulted in *L.*

monocytogenes being not detected or showing significant reductions in contamination load (172). Other studies have assessed how different bacterial species influence *L. monocytogenes* within the biofilm. Carpentier and Chassaing (173) reported the spatial arrangement of *L. monocytogenes* biofilms differed when grown in dual species biofilms, with isolates that increased *L. monocytogenes* biofilm counts compared to single cell mono-species biofilms. The three Gram-positive strains tested formed microcolonies, and *L. monocytogenes* gathered around these structures. Whereas *L. monocytogenes* isolates formed their own microcolonies when grown with the two Gram-negative isolates (173). In dual-species culture of a non-antagonistic producing strain of *Bacillus cereus, L. monocytogenes* biofilm counts were greater compared to *L. monocytogenes* mono-biofilms and the two bacterial species were observed to coexist surrounded by a small amount of EPS (169). While the importance of identifying bacterial species which have an antagonist effect against *L. monocytogenes* will allow for the development of alternative control measures, attention and understanding on bacterial species which support the growth and survival within biofilms is also warranted to further our knowledge on how *L. monocytogenes* is able to interact and persist within the FPE.

Biofilm related genes

Replication of the FPE is a difficult task due to the various elements and conditions present, therefore an alternative solution to understanding *L. monocytogenes*' ability to persist within the FPE is to identify potential genes which may be involved in the biofilm and colonisation processes. Various studies have attempted to elucidate the genetic determinants responsible for biofilm formation in *L. monocytogenes* through techniques including insertional transposon mutagenesis and genome wide association studies, as examples (174, 175). Some of the main molecular determinants (Table 6) reported to play a role in *L. monocytogenes* biofilm formation include the production of flagella at temperatures under 30 °C, which is involved in surface attachment in the early stages of biofilm formation. Isolates with mutations in the flagella genes demonstrated a 10-fold decrease in surface colonisation (176). The *agr* cell to

Table 6. Key genetic determinants purported to be involved in biofilm formation in *L. monocytogenes*.

Biofilm determinant	Gene function	Reference
Flagella genes	Surface attachment in early stage biofilm formation below 30 °C	(176)
agr system	Cell to cell communication system – early stage biofilm formation and abiotic adherence	(177, 178)
luxS	*luxS* negatively regulates attachment and biofilm formation with mutants producing denser biofilms	(179)
prfA	Flagella transcription regulation and biofilm maturation are some of the roles the virulence regulator has	(180)
sigB	Class II stress response regulator, may have multiple roles in biofilm formation	(181)
bapL	Biofilm-associated protein; cell wall anchored protein associated with abiotic adherence	(182)
relA *hpt*	Both genes required for synthesis of (p)ppGpp for growth after adhesion in limited nutrient conditions	(183)
dltAB	Responsible for D-alanylation of lipoteichoic acids involved in cell surface charge and adhesion to abiotic surfaces.	(184)

cell communication system is also involved in early stage biofilm formation and adherence to abiotic surfaces (177, 178). In contrast, the communication autoinducer 2 precursor coded by *luxS* was shown to negatively regulate attachment and biofilm formation, with *luxS* mutants producing denser biofilms (179). The virulence regulator *prfA* was also shown to regulate flagella transcription as well as influence biofilm maturation (180). The class II stress response regulator *sigB* has been shown to be highly expressed during static and continuous flow biofilms; in addition, *sigB* regulates a large array of genes, and therefore the *sigB* regulon may also play multiple roles in biofilm formation (181). Other genes which have been reported to play a role in biofilm formation depending on the environmental conditions, or among a limited number of strains, have also been reported. The biofilm-associated protein, *bapL,* is a cell wall anchored protein shown to be involved in attachment to abiotic surfaces; however, it was not

present across the majority of isolated strains (182). Taylor et al (183) reported in nutrient starvation conditions, *relA* and *hpt* are necessary for the synthesis of (p)ppGpp, which is required for growth after adhesion. Various genes responsible for cell wall components have also been shown to play a role in adhesion and biofilm formation. The *dltA* and *dltB* genes responsible for D-alanylation of lipoteichoic acids are involved in cell surface charge and adhesion to abiotic surfaces (184). A study by Piercey et al (185) identified mutants in peptidoglycan and teichoic acid biosynthesis resulted in decreased biofilm formation. A vast array of genetic determinants either directly linked to biofilm formation like surface proteins, or indirectly to cellular growth or cell wall production, have been identified; however, as *L. monocytogenes* is able to switch between the saprophytic and pathogenic lifecycle, it holds a boundless genetic capacity that can be utilised in both lifestyles.

Stress survival of *L. monocytogenes* in food and the FPE

A key characteristic of *L. monocytogenes* is its ability to survive a variety of environmental conditions, which generally may place a bacterium under stress and discourage growth or survival. Within the FPE, production managers utilise a variety of these stress factors to help mitigate or reduce growth and survival (Table 7). These elements can be intrinsic (food-related) or extrinsic (intentionally applied to reduce microbial contamination and spoilage) and include high osmolarity, low and high temperatures and pH, disinfectants, sanitisers and episodes of desiccation (186).

Desiccation

The removal of moisture, also known as desiccation, to reduce the water activity of the surrounding environment through air drying is an extrinsic stress factor applied to inhibit the growth and survival of bacterial cells (187). Studies into *L. monocytogenes* strains ability to tolerate various levels of desiccation have suggested an increased survival ability within FPE (143, 188, 189). Vogel et al (187) reported *L. monocytogenes* cells, after initial decreases in

CFU/cm^2 within the first seven days, were capable of surviving long periods of desiccation (tested up to 91 days). The addition of nutrients from organic material or 5 % NaCl was capable of increasing desiccation survival and maintaining viable cells until favourable conditions returned and cells were able to return to active growth. The ability to form biofilms has also been shown to provide increased tolerance to desiccation, while also increasing the number of cells available for transfer to food products and therefore providing increased potential for cross contamination of food products (190).

Osmolarity

Salt, or NaCl, is predominately used as a preserving agent in food products and provides an osmotic stress to bacterial cells by affecting the water activity of the extracellular environment. *L. monocytogenes* is able to grow in high salt concentrations, up to 12.5 % w/v NaCl predominately as a result of the uptake of osmolytes to restore osmotic pressure and turgor to the cell (191-193). *L. monocytogenes* is not able to synthesise the required osmolytes and therefore relevant systems and genes are required to transport the compatible solutes from the extracellular environment (192). The carnitine transport system is mediated by the *opuCABCD* genes, and couples osmolyte transport and ATP hydrolysis together to provide energy to cross the cytoplasmic membrane (194). The glycine betaine transport system is mediated by two porter systems, *gbuABC* and *betL*. The glycine betaine porter I, *betL*, is a secondary transporter which transports glycine betaine along with a sodium ion and has been shown to be involved in the initial response to hyperosmotic conditions (192, 195). The second glycine betaine system consists of the *gbuABC* operon which encodes the glycine betaine porter II (gbu) transporter, which is initiated by osmotic stress and has been reported to be involved in long term osmotic stress adaptation (192, 196). While there are other genes with a role in the response to osmotic stress, the *betL*, *opuC* and *gbu* transporters are the main response systems.

Heat stress

The use of temperatures above optimal growth temperature (37 °C) is a common stress treatment used during food processing. While thermal treatment can prove to be advantageous in the inactivation of microbial cells, both of spoilage and pathogenic bacteria, temperatures which are too high can affect the nutritional quality and sensory attributes of associated foods (196). It has been noted that *L. monocytogenes* is more heat tolerant than other non-spore forming bacteria due to the bacterium's ability to survive within adverse environmental conditions, including at higher than optimal growth temperatures (196). Although thermal treatments are generally considered effective, the concern surrounds exposure to sublethal temperatures in which *L. monocytogenes* cells may develop an adaptation to the heat stress or an induced stress resistance (197). This is a particular concern for certain food products which require non-isothermal treatments to preserve their integrity. Monfort et al (198) found non-isothermal treatment of liquid whole egg provided 5-$\log_{10}$ reductions of *L. monocytogenes,* much lower than the 9-$\log_{10}$ reductions reported in the literature for pasteurisation of egg products, and suggested *L. monocytogenes* cells experienced heat shock during the heating lag phase thus providing protection during the subsequent higher temperatures. Pre- and post-processing storage temperature has also been shown to influence thermotolerance. Pagán et al (199) reported increased heat resistance by cells grown at 4 °C compared to 37 °C grown cells, as well as the ability to tolerate heat shock when grown at 30 °C first. During exposure to mild thermal temperatures bacterial species induce genes involved in heat shock response. Within *L. monocytogenes,* the heat shock response involves class I (*grpE*, *dnaK*, *dnaJ*, *groEL* and *groES*) and class III (*clpC*, *clpP* and *clpE*) heat shock proteins and the class II general stress response (*sig-B* dependant) at different stages of thermal treatment (196, 200), with the main function to prevent aggregation of proteins by stabilising and reassembling partially unfolded proteins due to heat stress. In addition, van der Veen also demonstrated the SOS response responsible for

the repair of damaged DNA is also activated during heat shock (200), demonstrating *L. monocytogenes* has numerous adaptive mechanisms towards thermal processing.

Cold stress

L. monocytogenes is capable of surviving and growing at refrigeration temperatures as low as -0.4 °C (24), making it a difficult bacterium to control. While generation times and growth rates at refrigeration temperatures generally become static in most bacterial species, *L. monocytogenes* isolates can continue replication and grow, albeit at a slower rate. While some studies have attempted to attribute tolerance of cold temperatures to persistence or isolation source (145, 201), it appears cold tolerance is inherent and the result of the expression of specific genes employed to counteract the negative effects that may occur at low temperatures. Cold shock proteins (csps), *cspA*, *cspB* and *cspD*, have been identified within *L. monocytogenes* and have been shown to play a major role in cold adaptation (202). The presence of csps allows for the destabilising of RNA secondary structures and the binding of single stranded nucleic acids, ultimately acting as DNA and RNA chaperones influencing replication, transcription and translation during cold stress (203). Along with csps, changes to branched change fatty acids and the uptake of osmolytes can provide cryotolerance or cryoprotection to cells at low temperatures (195). Growth stimulation of cold stressed cells can occur through Gbu-mediated betaine uptake, however along with *betL*, only offers a small degree of cryotolerance; nonetheless, these are still required during cold stress (195). In comparison, *opuC* mediated carnitine transport provides increased cryoprotection (192).

Acidic stress

Listeria monocytogenes has an innate ability to tolerate acidic environments within the FPE or gastric environment, to survive lower pH that is utilised as a preservation method, or to facilitate gastric transit and initiate the infection cycle (204). Similarly to the other FPE conditions mentioned above, *L. monocytogenes* can develop an adaptative acid tolerance response when exposed to mild acidic conditions resulting in a greater tolerance to lower

acidic conditions as demonstrated by Lou and Yousef (205); these authors demonstrated that *L. monocytogenes* cells acid adapted at pH 4.5-5 for one hour were able to resist pH 3.5, normally considered lethal. The *L. monocytogenes* genome also contains specific genes and systems designed to increase its survival within acidic conditions. The glutamate decarboxylase (GAD) system consists of three glutamate decarboxylase enzymes, *gadD1*, *gadD2*, *gadD3* and two glutamate/GABA antiporters, *gadT1*, *gadT2,* which ultimately relieve acidification of the cytoplasm (206). The F_0F_1 ATPase complex induces the acid tolerance response illustrated by Lou and Yousef (205), mentioned above. The cytoplasmic pH is elevated by the production of ammonium to neutralise intracellular protons through the arginine deiminase (ADI) and the agmatine deiminase (AgDI) systems. The above-mentioned systems and genes are some examples of ways *L. monocytogenes* maintains pH homeostasis.

L. monocytogenes response to FPE stressors is not specialised to the individual stressors; rather, a cross protection is provided with some systems providing increased tolerance when two stressors are activated. For example, the response to osmotic stress also induces tolerance to low temperatures (192). Lou and Yousef (205) demonstrated cells adapted to sublethal levels of acid or ethanol increased resistance to lethal concentrations of acid, ethanol and hydrogen peroxide; sublethal ethanol exposure increased resistance to NaCl; exposure to sublethal hydrogen peroxide, NaCl and heat increased resistance to lethal levels of hydrogen peroxide; and heat shock was associated with resistance to lethal levels of ethanol and NaCl. Faleiro et al (207) reported acid adapted and osmotic adapted *L. monocytogenes* isolates displayed cross protection to the high concentrations of the other stressor. Phan-Thanh et al (208) showed acid adapted *L. monocytogenes* cells displayed increased resistance to alcohol stress, osmotic shock and heat shock, along with heat adapted cells presenting increased resistance to acid shock conditions.

In addition, the presence of genomic islands also confers tolerance to a variety of stress conditions. For example, the presence of stress survival islet (SSI) -1 is associated with tolerance to acidic and osmotic conditions (209) whereas SSI-2 is correlated with alkaline and oxidative stress tolerance (210). The alternative sigma factor sigma B (*sigB*) regulates the class II general stress response in *L. monocytogenes* along with a large selection of *sigB* dependent genes involved in stress response and virulence (200, 211). The *sigB* general stress response has been shown to play a key role in resistance to osmotic, oxidate, acid and low temperature stress (212-215), and further, to the sublethal adaptation mechanism of *L. monocytogenes*. *L. monocytogenes*' ability to survive various processing stressors influences its ability to colonise and persist in the FPE, making it an important food borne pathogen.

Disinfectants

The control of *L. monocytogenes* within the FPE has for many years been associated with the application of disinfectants. When applied at the appropriate in-facility concentrations, disinfectants have an inhibitory effect on *L. monocytogenes*. However, subinhibitory levels may occur due to the following factors (62):

- insufficient removal of organic matter prior to cleaning and disinfection,
- application of disinfectant on surfaces or areas where there is pooled water or onto wet surfaces,
- dosage failures.

Exposure to subinhibitory levels can result in an adaption to applied disinfectants or create a selective pressure for the transfer of resistance genes. A commonly utilised disinfectant class within the FPE is quaternary ammonium compounds (QAC), which have a cationic mode of action affecting the cytoplasmic membrane of bacterial species (216). Benzalkonium chloride (BC) is a QAC based disinfectant commonly used within the FPE. Reports of disinfectant resistant strains of *L. monocytogenes* isolated from the FPE have been documented. Mullapudi et al (217) isolated 123 strains from three turkey processing facilities and found 46%

of isolates were resistant to BC. Ratani et al (218) also reported 13 % of isolates from food and FPEs were BC resistant. In comparison, Haubert et al (219) reported 100 % resistance of *L. monocytogenes* strains isolated from food and FPEs in Brazil. In addition to the observed phenotypic resistance, the ability to adapt to higher levels of disinfectants when exposed to subinhibitory concentrations has also been reported. Lundén et al (220) tested four different types of disinfectants and found two-hour sublethal exposure to three of the disinfectants (two QAC and a tertiary alkyamine) resulted in a three-fold increase in minimum inhibitory concentration (MIC). In addition, subsequent exposure to increasing concentrations of the above three disinfectants also resulted in a 15-fold increase along with the previously identified sensitive isolates displaying similar adaptive MICs to the resistant strains (220). Furthermore, exposure to sublethal concentrations of one type of disinfectant resulted in cross adaption to the other disinfectants even when they were from a different class, except potassium sulphate which although providing cross adaptation to the other disinfectants, exposure to the other disinfectants did not increase the resistance to potassium sulphate (220).

A variety of genetic determinants responsible for resistance to BC or other disinfectants have been reported. These include genes specific for resistance to BC and genes coding for efflux pumps which display a broader specificity. In *L. monocytogenes*, disinfectant resistance has been associated with the presence of transposons or efflux pumps located either chromosomally or on plasmids (11). Two chromosomal encoded efflux pumps, *mdrL* and *lde*, belonging to the major facilitator superfamily have been identified in *L. monocytogenes* strains. Jiang et al (221) showed the chromosomal *mdrL* efflux pump contributes to BC tolerance in the *L. monocytogenes* EGD-e strain, and when the efflux pump inhibitor, reserpine was added a three-fold decrease in MICs was observed. While the *lde* efflux pump has predominately been associated with resistance to ciprofloxacin (222) and fluoroquinolones (223), there are reports of low expression of *lde* in BC-resistant strains (224). A transposon identified on plasmid pLM80 described in *L. monocytogenes* strain H7550 was found to contain a disinfectant resistance cassette *bcrABC* (225). The *bcrABC* cassette consists of a

transcriptional regulator (*bcrA*) and two small multidrug resistant (SMR) family genes (*brcBC*) (225). Expression of *bcrABC* was found to be increased at sublethal levels as well as at low temperatures (225, 226) indicating strains containing the *bcrABC* cassette may have increased fitness within FPEs. Minarovičová et al (227) found *L. monocytogenes* strains positive for the *bcrABC* cassette displayed the highest BC MICs, which was the result of the *bcrABC* expression. An integrated transposon identified in *L. monocytogenes* strains 6179 and 4423 was reported to confer resistance to BC and other QACs as a result of a SMR transporter, *qacH* (228, 229). The presence of *qacH* was associated with higher MICs (228) and has been detected in various food processing industries including meat, fish, RTE products, FPEs and as well as clinical isolates (216, 230). The *Listeria* genomic island 1 identified in strain 08-5578 was found to also confer resistance to QACs as a result of a SMR family efflux pump, *emrE* (231). The *emrE* gene is the least common of the BC resistance genes present within the *L. monocytogenes* population reported to date and appears at this stage to be associated with clonal complex 8 (230). While the application of disinfectants at the appropriate concentrations is sufficient, the potential for resistance as well as cross adaptation to other disinfectants, antibiotics, FPE stressors and the limited effectiveness against biofilms warrants additional strategies in the control of *L. monocytogenes*.

Table 7. Some of the key genetic determinants associated with a selection of FPE stressors utilised to control *L. monocytogenes*.

FPE stressors	Key genetic determinants	References
Osmolarity	opuCABCD, gbuABC, betL, SSI-1	(192, 194-195)
Heat Stress	grpE, dnaK, dnaJ, groEL, groES, clpC, clpP and clpE	(196, 200)
Cold stress	cspA, cspB, cspD, betL and opuC	(192, 195)
Acidic stress	gadD1, gadD2, gadD3, gadT1 and gadT2, SSI-1	(206)
Disinfectants	mdrL, lde, bcrABC, qacH, emrE (LGI1)	(221-222, 225, 228-229, 231)

Biocontrol

The application of biological methods to control *L. monocytogenes* has the potential to alleviate some of the tolerance, adaptation and cross adaptation concerns from the use of disinfectants and food related stressors, particularly against the development and eradication of biofilms. Utilisation of beneficial microbes, their antimicrobial products and plant derived products, has the potential to offer an alternative, synergist or supplementary mitigation method. Biocontrol methods with potential to act against listerial biofilms include bacteriophages and their endolysins, competitive bacterial species and their antimicrobial products bacteriocins, and plant-derived products. The utilisation of the most abundant microorganisms, bacteriophages (phages), is not a new phenomenon. Their ability to infect bacteria, particularly against hard to treat bacteria, was initially used in clinical applications as far back as the 1920s (232). Renewed interest from a food safety perspective in recent decades has occurred with the successful development of two commercial bacteriophage products, ListShield™ and Listex™ P100, approved for use as food preservatives (232-234). Reductions ranging from 1.4-2.3 log CFU/g at temperatures of 4, 10 and 22 °C have been reported for Listex™ P100 when applied to catfish fillets (234). In low contaminated dry cured ham, ListShield™ was able to reduce counts to below detection levels, and in highly contaminated samples a 3.5 log CFU/cm^2 reduction was observed (235). However, the efficacy of in-facility phage treatments against biofilms are of specific interest to food processing managers. For phages to be successful in the lysis of bacterial cells they produce hydrolytic enzymes called endolysins (lysins) (236). Lysins can be harnessed through protein expression and purification and then applied externally to food products or surfaces targeting the bacterial cell wall (237). The use of antagonistic bacterial species to compete against target bacteria over space or nutrients is another biocontrol method. Competitive bacterial studies can be divided into three types of strategies: competition, where planktonic cells of both species are co-cultured for a period of time; exclusion, where the antagonistic species are grown to a predetermined cell density prior to the addition of planktonic cells of the target

species; or displacement, in which the target species are grown to predetermined cell density prior to addition of planktonic antagonists (238, 239). The production of antimicrobial products, particularly bacteriocins can also be utilised as a biocontrol method. Specificity varies depending on the bacteria producing the bacteriocin, with some bacteriocins displaying a narrow spectrum while others have a broad spectrum of activity encompassing members within the same genus as well as other genera and species (240). Similar to lysins, bacteriocins can be extracted and applied as either a crude or semi-purified product (241). The isolation of a bacteriocin, nisin, from *Lactococcus lactis* is an example of the successful identification and application as a commercial food preservative against Gram-positive bacteria including *L. monocytogenes* (242). An alternative to the use of microorganisms as biocontrol options is the use of plant-derived antimicrobials, essential oils (EOs). Thousands of EOs have been described with around 300 of these recognised as safe for use as preservatives (243). While the antimicrobial properties of EOs have been purported in natural medicines for many years, their applicability in food is still undetermined as they may require use at concentrations that may impart negative sensory effects (243).

While good success of alternative antimicrobials has been shown against planktonic cells, particularly bacteriophages which have had commercial success as a food preservative, further research is required on biofilms at various stages. Initial reports have indicated promising results across all biocontrol options; however, some draw backs have been identified particularly surrounding the utilisation of lysins and bacteriocins, which generally required additional research and development. Furthermore, the applicability of EOs also requires substantial research. Understanding the microbial context of the FPE and utilising competitive bacterial species or their antimicrobial products can provide increased control and individualised treatment of *L. monocytogenes.* However, it generally requires an initial outlay by the food producer to gain this insight, which may limit small processing facilities from being able to access the most applicable biocontrol method for their facility. Nonetheless, the utilisation of biocontrol methods as a novel approach warrants further attention; particularly

surrounding their effectiveness against multi-species biofilms, conditions reflective of the FPE and in-facility application.

Thesis objectives

The ability of *L. monocytogenes* to colonise, survive and persist within various FPEs irrelevant of the various hurdles and stresses present makes *L. monocytogenes* an important foodborne pathogen of concern. The overall aim of this thesis was to improve the current knowledge surrounding *L. monocytogenes* ability to colonise surfaces and survive at conditions reflective of the FPE in addition to assessing the isolates fitness, virulence and therapeutic potential.

The objectives of this research are to:

1. **Develop a high-throughput screening of biofilm formation of *L. monocytogenes* on stainless steel coupons using a 96-well plate format.**

A model biofilm system was developed to assess biofilm formation under conditions reflective of the FPE including low temperature and nutrient conditions against a common surface material present within processing facilities. A high-throughput method was developed to effectively screen up to 52 isolates over a 96-h period.

2. **Increase understanding surrounding the colonisation dynamics of *L. monocytogenes* strains isolated from food production environments.**

The ability to form biofilms utilising the model biofilm system was determined to identify fast and slow biofilm formers. Selected isolates were assessed on a variety of factors potentially influencing survival within the FPE, including expression of a signalling peptide, growth rate, EPS production and differences in transcription.

3. **Perform a phenotypic and genomic analysis of *L. monocytogenes* for their fitness and virulence potential utilising a selection of key factors.**

The aim of this research area was to characterise *L. monocytogenes* isolates from food and environmental sources using phenotypic and genomic methods, to determine their ability to survive within the food production environment, their potential to cause infection and their susceptibility to frequently used antibiotics in the treatment of listeriosis

4. **Evaluate the novel biocontrol methods for *Listeria monocytogenes* biofilms in food production facilities.**

Listeria monocytogenes is a difficult microorganism to prevent from entering and persisting within the FPE. The adaptability to the various environmental conditions with the presence of multiple antimicrobial resistance mechanisms has seen the persistence of this bacterium increase therefore additional measures are required to aid in mitigation. The use of bacteriophages, endolysins, competitive bacterial species, bacteriocins and plant-derived antimicrobial products, are reviewed as alternative control options.

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CHAPTER 2

High-Throughput Screening of Biofilm Formation of *Listeria monocytogenes* on stainless steel coupons using a 96-well Plate Format.

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Abstract

Listeria monocytogenes is a food borne pathogen capable of colonising and persisting in the food production environment (FPE). While there are a variety of factors involved in *L. monocytogenes* ability to persist in FPE, the ability to form biofilms has the potential to increase their chance of survival and long-term colonisation. Understanding the mechanisms involved in *L. monocytogenes* ability to form biofilms may potentially help food safety managers optimise control strategies targeting them in the FPE. In this chapter, a high throughput method to determine *L. monocytogenes* ability to attach and form biofilms utilising FPE-grade stainless steel is described. This method provides fast and efficient results, facilitating scaling up to large numbers of isolates to measure their ability to form biofilms, where lower throughput approaches can then be utilised to further characterise isolates of interest.

Introduction

Colonisation and persistence of microorganisms in the food production environments (FPEs), particularly bacterial foodborne pathogens such as *Listeria monocytogenes*, can be a serious concern for food safety managers. Once introduced into the FPE, *L. monocytogenes* can colonise niches through a combination of genetic and environmental mechanisms. The ability to attach quickly and form biofilms may greatly increase a bacterial species survival in the FPE. Biofilms are microbial cells attached to each other or a surface surrounded by an extracellular polymeric substance (1, 2). By living within a biofilm community, the various species increase their ability to survive environmental stressors as they are protected from desiccation, the antimicrobial action of disinfectants and toxic metabolites along with being provided increased opportunity for horizontal gene transfer, a constant supply of shared nutrients and increasing the biofilm formation of poor biofilm formers (3, 4). Due to the increased survival capacity biofilm formation provides *L. monocytogenes* in the FPE, it is important to understand the various mechanisms involved. Previous techniques may be divided into static methods (nutrients are supplied during inoculation and remain for the whole experiment), or semi-continuous or continuous (nutrients are supplied at various intervals or rates and the spent media is removed) (5). Semi-continuous or continuous biofilm models have included flow cells (6), drip flow biofilm reactor (7) and the Centres for Disease Control biofilm reactor (8) which are able to replicate the development of biofilms in the natural environment however only a small number of isolates are able to be analysed at once. In comparison, static micro-titre plate-based assays (9, 10) have the capacity to process a high number of isolates at once however they typically do not replicate the FPE as accurately. Being able to replicate conditions similar to the FPE will allow for the data to be translated into relevant information for food safety managers. Analysing bacterial strains ability to form biofilms against materials relevant to the FPE including glass, wood, plastic and stainless steel increases the understanding of how particular strains are capable of persistence. A high throughput method to determine *L. monocytogenes* ability to form biofilms utilising stainless

steel coupons is described below. This method provides fast and efficient results, scaling to large numbers of isolates; this can identify isolates of interest for further analysis with lower throughput approaches such as semi-continuous or continuous systems, providing a more comprehensive understanding of biofilm formation dynamics. In addition, the method can be utilised for sanitiser/disinfectant assays or multispecies competitive exclusion assays.

Materials

1. *Listeria monocytogenes* cultures
2. Stainless steel coupons, type 304, mill finish, 5 mm (diameter), 0.9 mm (height) (Figure 1)
3. 96 well micro-titre plates – U bottom and Flat bottom
4. Brain Heart Infusion (BHI) broth or other non-selective
5. BHI agar plates
6. Sterile water
7. Maximum recovery diluent (MRD), sodium chloride 8.5g/L and peptone 1.0 g/L
8. Sonicating water bath
9. Parafilm
10. Sodium hydroxide, 3%
11. Peracetic acid (PAA), 0.1%
12. Ethanol, absolute
13. Tweezers
14. Bunsen Burner
15. 1.5 mL microfuge tubes
16. Rack for microfuge tubes
17. Programmable incubator

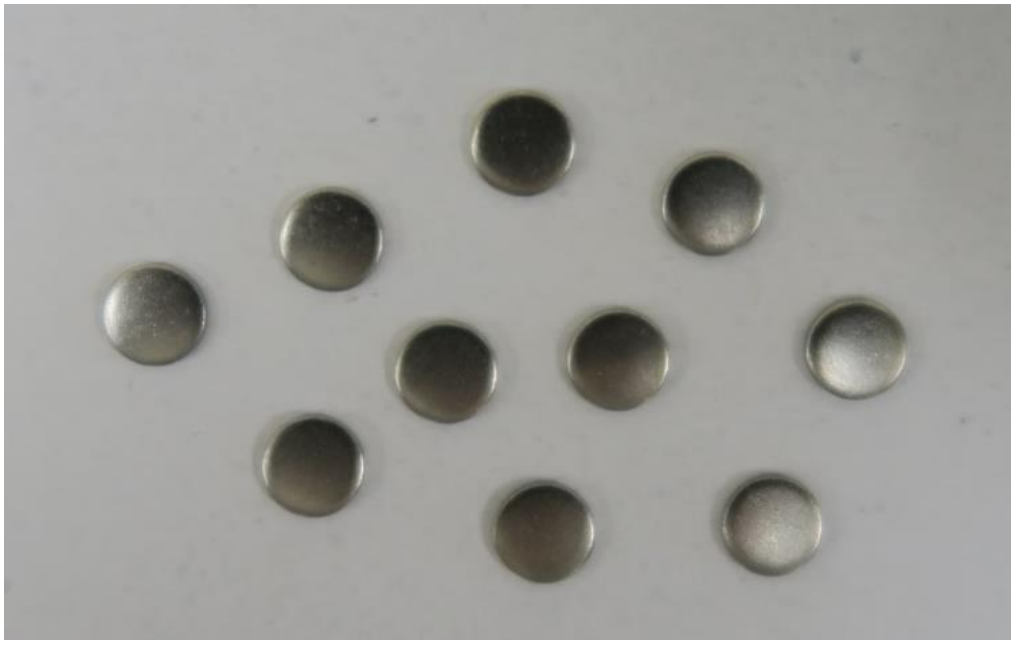

Figure 1. Stainless steel coupons which fit inside 96 well micro-titre plates.

Methods

Preparation of cleaning solutions

1. Stainless steel coupons are cleaned by soaking in NaOH solution for 20 mins.
2. Rinse coupons three times with DI water.
3. Disinfect coupons by soaking in PAA for 2 mins.
4. Rinse coupons with DI water three times, then autoclave at 121 °C for 15 mins. Dry autoclaved coupons in preparation for use.

Preparation of media and solution

1. Prepare BHI broth, agar and MRD as per suppliers' instructions.
2. Prepare sterilised deionised water.
3. Dilute BHI broth to a 1:10 solution (dBHI; *see* Note 1).

Prepare overnight cultures of L. monocytogenes isolates.

1. Overnight cultures of each strain to be tested should be prepared by growing in BHI for 18 h at 37 °C (*see* Note 2).

	1	2	3	4	5	6	7	8	9	10	11	12
A	Isolate 1		Isolate 2		Isolate 3		Isolate 4		Isolate 5		Isolate 6	
B	Isolate 1		Isolate 2		Isolate 3		Isolate 4		Isolate 5		Isolate 6	
C												Control
D	Isolate 7		Isolate 8		Isolate 9		Isolate 10		Isolate 11		Isolate 12	
E	Isolate 7		Isolate 8		Isolate 9		Isolate 10		Isolate 11		Isolate 12	
F												Control
G	Isolate 13		Isolate 14		Isolate 15		Isolate 16		Isolate 17		Isolate 18	
H	Isolate 13		Isolate 14		Isolate 15		Isolate 16		Isolate 17		Isolate 18	

Figure 2. Example of position of isolates and stainless-steel coupons in 96 well U bottom micro-titre plate for biofilm formation assay.

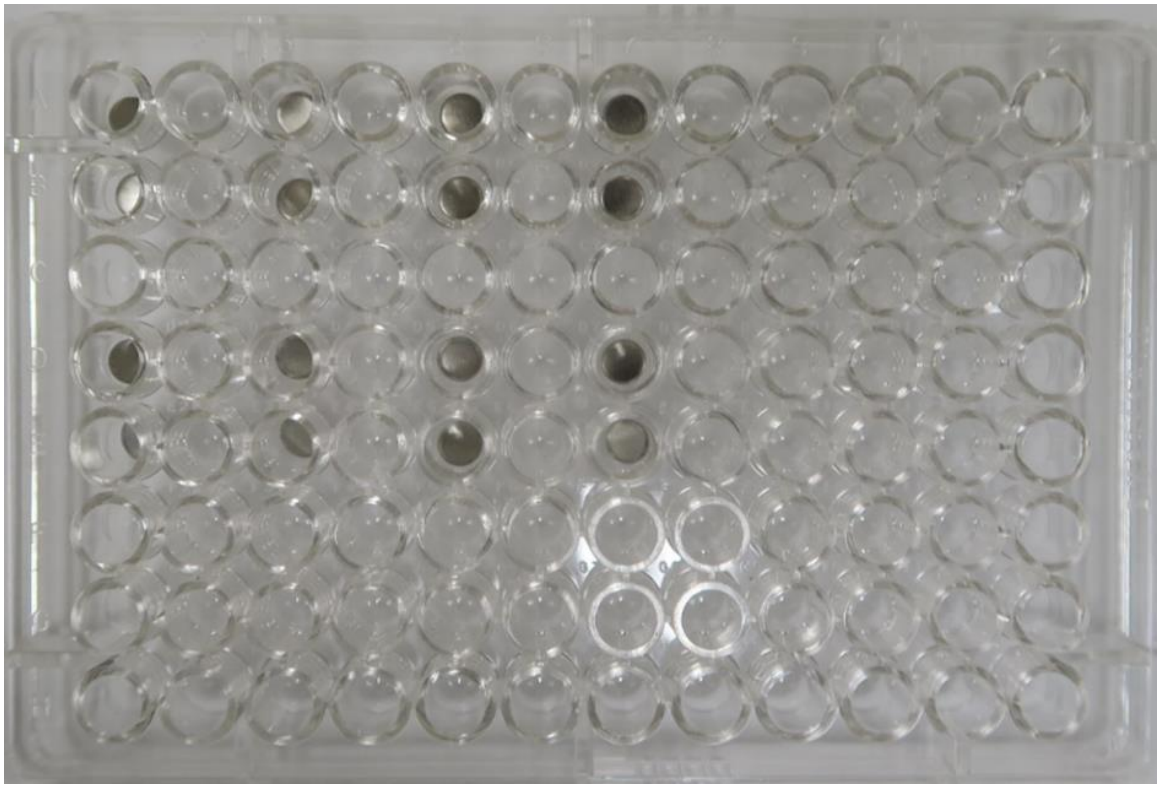

Figure 3. Stainless steel coupons in micro-titre plate.

Preparation of microplates

1. Flame sterilise a pair of tweezers using absolute ethanol, and without re-contaminating the tweezers place 1 stainless steel coupon into each well of the 96-well microtitre plate which will be used (*see* Note 3; Figure 2 and 3).
2. Add 50 µL of dBHI to each well with a stainless-steel coupon.
3. Prepare dilutions of overnight cultures using dBHI to 10^2 CFU/mL (*see* Note 4)
4. Add 100 µL of the 10^3 dilution to each test well containing a coupon.
5. Prepare 4 micro-titre plates using the same plate setup (*see* Note 5).
6. Place micro-titre plates into an incubator set to 14 °C, check the time when placing in the incubator, this is the target time point and the coupons should be processed within 1 hour of this time point.

7. Plate 100 µL of the 10^3 and 10^2 dilutions on duplicate plates to enumerate starting cell numbers at time 0.

Processing of coupons

1. Check the inoculation plates to make sure the appropriate cell concentration was inoculated.
2. At the appropriate time points (e.g. 24, 48, 72 or 96 hours) remove the microplate allocated to that time point from incubator.
3. Remove the spent media, discard (*see* Note 6)
4. Place a new 96 well microplate (with no coupons in it) on top of the plate with coupons in it, turn both plates upside down to transfer the coupons to the new plate (*see* Note 7 and Figure 4).
5. Wash the coupons with 200 µL of sterile water three times (*see* Note 8).

Figure 4. Place new micro-titre plate on top of plate with coupons, flip upside down so the new micro-titre plate is on the bottom and give a tap to transfer coupons to new micro-titre plate.

6. Add 240 µL of MRD to each well which has a coupon in it, place lid on plate and wrap sides with parafilm (Figure 5).

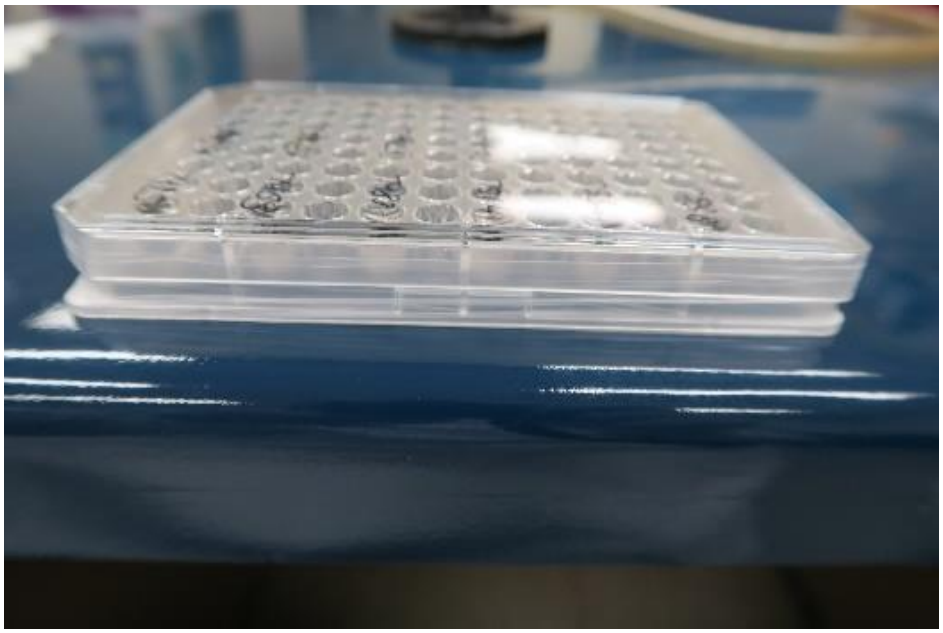

Figure 5. Wrap parafilm around base and lid of micro-titre plate prior to sonication to prevent contamination with sonicating water batch medium.

7. Sonicate for 5 mins to detach cells from coupons (see Note 9).
8. In a flat bottom micro-titre plate add 270 µL of MRD per sonicated well (see Note 10).
9. Take 30 µL of sonicated MRD and perform a serial dilution in the flat bottom micro-titre plate
10. For the 24 h micro-titre plate, plate 100 µL at the appropriate dilution (see Note 11).
11. Incubate plates for 24 hours
12. The next day count the CFUs on each plate and calculate CFU/cm^2.
13. Follow the same process (from 3.5) for the other plates at the appropriate time points (48, 72 and 96 h).

14. Cell numbers should be expressed on log scale, as colony forming units per cm^2 (as calculated based on coupon size).

Notes

1. The 1:10 BHI growth medium can be substituted with another growth medium as required (e.g. for growth in a nutrient-rich medium, undiluted BHI can be used).
2. 5 ml overnights are sufficient.
3. It is suggested a technical replicate is performed for each isolate therefore two wells with a stainless-steel coupon in each should be allocated to 1 isolate (Figure 2 and 3).
4. It is suggested to inoculate the assay at 10^3 for any attachment and biofilm formation to not be the result of a high inoculation number. Preparing the extra dilution 10^2, will determine the number of CFUs which were actually plated.
5. For four time points (e.g. 24, 48, 72 and 96 h), prepare four plates. This can be adjusted as required, to meet the appropriate number of time points to be tested.
6. When removing the spent media be careful not to disturb the cells attached to the coupons; you may find tilting the plate slightly will allow you to remove as much spent media as possible.
7. It is important to hold the plates together as tightly as possible to prevent the plates from separating and the coupons moving. It is also important to remember that when transferring the coupons to the new plate that they will be in reverse order to the original plate. It is best to have a diagram of the new layout to assist with making the serial dilutions.
8. When washing the coupons, it is important to not aliquot the water directly onto the coupons to prevent the detachment of any cells prior to sonication.
9. Coupons should be sonicated for 5 to 10 minutes (do not exceed 10 minutes).
10. It is suggested serial dilutions for plating are prepared in a new flat bottom microplate, this will decrease the time required to prepare and mix the dilutions by using a multichannel pipette (Figure 6).

11. The number of dilutions required will depend on the individual isolates used. At 24 h it is suggested to plate directly from the coupon (neat) and either 10^{-1} or 10^{-2}. A trial run with a variety of dilutions plated will indicate which dilutions are appropriate.

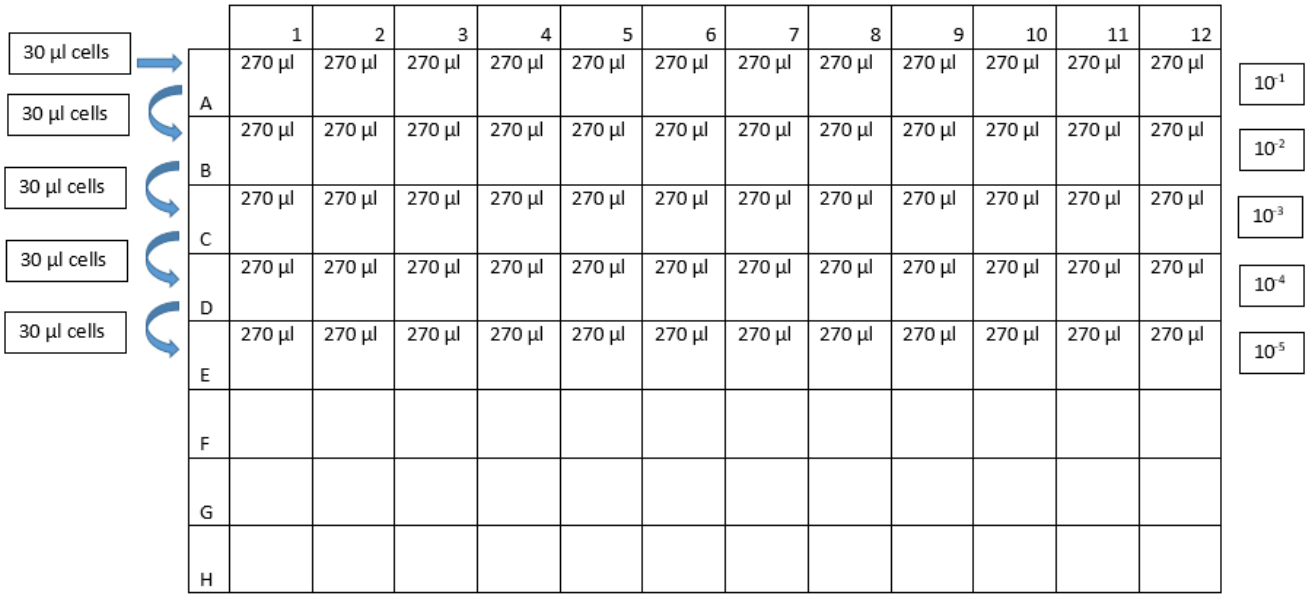

Figure 6. Example of how to perform serial dilutions using the 96 well flat bottom micro-titre plate.

*This method was developed for use in chapter 3; the results, strengths and limitations can be viewed there.

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CHAPTER 3

Colonisation dynamics of *Listeria monocytogenes* strains isolated from food production environments

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Abstract

Listeria monocytogenes is a ubiquitous bacterium capable of colonising and persisting within food production environments (FPEs) for many years, even decades. This ability to survive and persist within the FPEs can result in food product cross-contamination, including vulnerable products such as ready to eat food items. Various environmental and genetic elements are purported to be involved, with the ability to form biofilms being an important factor. In this study we examined various mechanisms which can influence colonisation in FPEs. The ability of isolates (n = 52) to attach and grow in biofilm was assessed, distinguishing slower biofilm formers from isolates forming biofilm more rapidly. These isolates were further assessed to determine if growth rate, exopolymeric substance production and/or the *agr* signalling propeptide influenced these dynamics and could promote persistence in conditions reflective of FPE. Despite no strong association with the above factors to a rapid colonisation phenotype, the global transcriptome suggested transport, energy production and metabolism genes were widely upregulated during the initial colonisation stages under nutrient limited conditions. However, the upregulation of the metabolism systems varied between isolates supporting the idea that *L. monocytogenes* ability to colonise the FPEs is strain specific.

Introduction

Listeria monocytogenes is a Gram-positive foodborne pathogen which can cause the life-threatening disease listeriosis, particularly in at-risk populations. While listeriosis is an uncommon foodborne illness, in the at-risk population group covering immunocompromised, elderly, pregnant women and neonates, the mortality rate can reach as high as 30 % (1-3). As the food supply chain has become progressively more global, increased reports of multistate and international food recalls and outbreaks are occurring, therefore the need to understand *L. monocytogenes* ability to colonise and persist in food processing environments (FPEs) is paramount (4). Traditionally the presence of *L. monocytogenes* in food products has been associated with foods like ready to eat meats, seafood products, unpasteurised milk and dairy products, however new food items like melons, various fresh, pre-cut and frozen fruit and vegetables, leafy greens, sandwiches and wraps are now being linked to *L. monocytogenes* (4). The ubiquitous nature of this foodborne bacterium makes it difficult to control and manage, and due to this can be repeatedly introduced into FPEs (5) and therefore efforts should be targeted towards this environment. It is not uncommon for reports of persistent strains to arise with studies describing the isolation of some strains over numerous years (6-9). The presence of persistent strains in the FPE can act as a repetitive source of contamination and imply the cleaning and sanitation program is not always effective in their control. Persistence within the FPE is suspected to be linked to a variety of factors including resistance and tolerance to disinfectants, acid and heat applications, favourable niches due to poor facility design and condition, along with the ability to attach to a variety of surfaces and the formation of biofilms (10-13).

Biofilms consist of microbial cells, generally multi-species, attached to each other or a surface, and surrounded by an extracellular polymeric substance which provides increased fitness to all cells within the biofilm (14-16). Biofilms provide increased protection from cleaning agents, disinfectants and desiccation, enhance the transfer of nutrients and removal of toxic

metabolites, and increase the opportunity to acquire new genetic traits (14-16). The process of attachment and biofilm formation in *L. monocytogenes* has been reported (17-19), however there is less consensus on what specific genes are responsible for *L. monocytogenes* ability to colonise and survive in FPEs, and it is likely that a synergy of multiple mechanisms are involved. The aim of this study was to develop a model system to reflect the FPE in terms of contact surface, temperature and limited nutrient availability, key conditions in the FPEs. This model system was then used to determine: i) if there were any differences in the early stages of biofilm formation between *L. monocytogenes* strains isolated from various food and environmental sources for multi-locus sequence types (MLST) commonly associated with FPEs; ii) if there are genes or phenotypes associated with the biofilm phenotype; iii) if there are differences in expression levels of the signalling associated *agrD* gene, known to be involved in adherence, between fast and slow biofilm formers; and iv) if there are differences in transcription levels of genes between two MLST STs both present in the slow and fast biofilm groups.

Methods

Bacterial isolates, culturing conditions and subtyping

A total of 52 *L. monocytogenes* isolates from 12 MLST subtypes (ST) (up to 5 isolates per ST) commonly associated with FPEs, and previously isolated from a variety of sources (i.e., dairy, meat, vegetable, mixed food and environment; Supplementary Table 1; appendix A) were chosen. Isolates were removed from - 80°C storage and resuscitated on Brain Heart Infusion (BHI, CM1136, Oxoid, UK) agar at 37°C for 24 h.

Stainless Steel coupons

Stainless Steel (SS) coupons of grade 304, mill finish (5 mm diameter by 0.9 mm thick; surface area 0.53 cm^2) were utilized. Coupons were cleaned in a solution of 3 % sodium hydroxide (Sigma-Aldrich, 72068, Australia) for 20 min, then 0.1 % peracetic acid (Oxysan, C16620,

Australia) for two minutes. Coupons were rinsed with sterile water three times between washes and then sterilised in the autoclave.

Biofilm formation analysis

L. monocytogenes isolates were grown for 18 h (+/- 1 h) in BHIB at 37°C. A high throughput biofilm screening method, previously developed (20), was used to determine the fastest and slowest biofilm forming isolates. Briefly, micro-titer plate wells containing SS coupons were inoculated aseptically with 100 µL of 10^3 CFU/mL in 1:10 diluted BHI (dBHI) and incubated at 14°C for 24, 48, 72 or 96 h (+/- 1 h) statically. After the appropriate incubation period the spent medium were removed, SS coupons were transferred to a sterile microtiter plate and underwent three rinses with sterile water. Coupons were sonicated in wells with Maximum Recovery Diluent (MRD; Oxoid, Thermo Scientific, Australia) for 5 min then 100 µL was serially diluted and plated onto BHI agar (BHIA) for enumeration at 37°C for 24 h prior to counting. Two biological replicates each with two technical replicates were performed on all 52 isolates, with an additional two biological replicates, again with two technical replicates, performed on 10 isolates. These 10 isolates comprised those with the fastest (*n*=5) or slowest average biofilm cell numbers after 24 h (referred to as the B10 isolates).

Genome wide association study

A genome wide association study (GWAS) was performed using the R package treeWAS (31) to identify genetic variants potentially responsible for variances in the biofilm phenotype at 24 h utilising a phylogenetic method accounting for population structure and recombination. Kchooser and Ksnp3 (32) was used to generate the optimal kmer value and core SNP matrix file from the biofilm isolates genome sequences.

Growth rate determination

Growth curves were constructed for the planktonic B10 isolates at 37°C in undiluted BHI and at 14°C in dBHI. For the growth curves, a single colony of each B10 isolate was inoculated in

5 mL BHI at 37°C at 150 rpm for 18 h (+/- 1 h). For the 37°C growth curve, 200 µL of a 1:200 dilution was aliquoted into a 96 well micro-titer plate and growth was monitored for 12 h at OD_{600} using an EON microplate spectrophotometer Gen5 (BioTek, Australia). For the 14°C growth curves, a 1:200 dilution of the 18 h (+/- 1 h) culture into dBHI was aliquoted into conical flasks and growth was monitored every 4 h until timepoint 15 h when growth was measured every 2 h at OD_{600} for 31 h. Maximum growth rate (µ) and doubling times (t_d) (2) were determined during the exponential growth phase using the equations: $\mu = (\ln OD_2 - \ln OD_1)/(t_2 - t_1)$ and $t_d = 0.693/\mu$, respectively, where ln refers to the natural logarithm, OD_2 is the late exponential phase OD, OD_1 is the early exponential phase, t_2 is time in minutes for OD_2 reading and t_1 is time in minutes for OD_1 reading, t_d is doubling time and µ is growth rate.

EPS production

Exopolymeric substance analysis was performed as follows: lysogeny broth (LB) agar without salt supplemented with 40 µg/mL Congo Red (CR) and 20 µg/mL Coomassie Brilliant Blue (CBB) was spotted with 5 µL of the 18 h (+/- 1 h) culture and incubated at 14°C for 48-72 h and 37°C for 24-48 h. After incubation plates were visually assessed to determine colony colour. For the CR assay, 18 h (+/- 1 h) cultures were grown in LB without salt and Muller Hinton broth at 37°C and 150 rpm. The CR and CBB assay were used to assess if there was production of any EPS substances (proteins, extracellular DNA, polysaccharides, exopolysaccharides or amyloid fibres).

Large batch biofilm formation

The biofilm process was upscaled for RNA extractions at 24 and 48 h for the B10 isolates. The biofilm process followed the initial screening experiment with the following changes: two coupons (15 mm × 15 mm × 0.55 mm) were used per isolate and time point; coupons were transferred to a new 70 mL container for three washes with DEPC-treated molecular grade water prior to biofilm removal with a cell scraper then sonication for 5 min. Cell scrapers were

vortexed briefly for 10 s then pulse vortexed five times to remove any attached cells. Cells were pelleted at 7000 × *g* for 10 min.

Total RNA extraction

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Australia) with the following adjustments: 25 mL of spent medium (SM) was collected from each time point and 1 mL of *Escherichia coli* DH5α was added as a carrier to assist in centrifugation of pellet. For the coupons, 2 mL of *E. coli* DH5α was used. RNA stabilisation was performing using a 5 % phenol ethanol solution as per Bhagwat et al 2003 (21). Enzymatic lysis of cells consisted of 100 units of mutanolysin (Sigma-Aldrich/Merck Australia) for 15 min in a 22°C water bath followed by 20 µL of 20 mg/mL Proteinase K for an additional 15 min. During RNA purification the spin column was washed twice with Buffer RW1. RNA yields were assessed on a Nanodrop device ND-1000 (Nanodrop, Thermo Fisher, Australia) and RNA quality assessment was performed on 2200 TapeStation System (Agilent, Australia) using high sensitivity RNA screen tapes. Samples were stored at -80°C until required for real time qPCR or and RNA sequencing.

Real time qPCR

DNase treatment and cDNA synthesis were performed on 1 µg of RNA using the iScript gDNA clear cDNA synthesis kit (Bio-rad, Australia) as per manufacturer's protocol. Real time qPCR (RTi-qPCR) was performed targeting the propeptide *agrD* as the gene of interest and *rpoB* as the housekeeping normalisation gene on the AriaMX Real-time PCR System (Aglient). Primer sequences were designed using primer3 in Geneious (2018) (Table 1). RTi-qPCR amplification was performed in 20 µL reactions with the mix containing 10 µL iTaq universal SYBR green Supermix (Bio-rad), 1 µL forward and reverse primers, 6 µL molecular grade water (Sigma-Aldrich) and 2 µL cDNA. PCR conditions were as follows: 3 min at 95°C followed by 40 cycles at 5 s at 95°C and 45 s at 60°C. Assays included a non-template control and non-reverse transcriptase for sample control with three biological replicates each with three technical replicates. Relative expression was determined using the $2^{-\Delta\Delta}$CT method (22). Stata

(Stata 15.1, StataCorp, College Station, Texas, USA) was used for statistical and data analysis. The nonparametric Wilcoxon rank sum test was performed on independent samples and the Wilcoxon signed rank test was performed on paired samples (p value < 0.05).

Table 1. Real time-PCR primer sequences designed for this study.

Primer set	Oligonucleotide sequence 5' → 3'
agrD-F	CAGTTGGTAAATTCCTTTCTAGAAAAC
agrD-R	TTTTCACAAATGGACTTTTTGGTTCG
rpoB-F	TGGGGCAGAACGTGTTATCG
rpoB-R	CCCACGGTTAGGGATGACAG

RNA sequencing and analysis/Transcriptomics

Four biofilm isolates from two STs which had an isolate in both the fast and slow biofilm formation groups were chosen for RNA sequencing. Total RNA extracts for sequencing were measured using the Qubit RNA high sensitivity kit (Thermo Fisher) and RNA extracts were sequenced by Western Sydney University Next Generation sequencing facility (NSW, Australia). Zymo-Seq RiboFree Total RNA Library Prep kit was used for rRNA depletion following the manufacturer's protocol. Depleted RNA samples were clustered on cBot and sequencing was performed as 2 × 125 bp paired end TrueSeq Cluster kit v4 and HiSeq SBS v4 kit on the Illumina HiSeq 2500 platform.

Sequence alignment was performed in Galaxy Australia (23) using the following tools: reads were mapped to each isolates draft genome sequence using BWA-MEM (Galaxy v0.7.17.1) (24), JBrowse genome browser was used to view the mapped reads (Galaxy v1.16.4+galaxy3) (25), SAM/BAM to count matrix using HTSeq code (v0.5) was used to produce differential

gene expression (DGE) count matrices. Gffread (Galaxy v0.11.6.0) was used to convert .gff3 files from the Patric database (26, 27) to .gtf files for count analysis. The log_2 counts per million for the DGE count matrix were determined by Voom/Limma in Degust (v4.1.1) (28). Individual isolate comparisons consisted of 7453 24 h with 7453 48 h, 7545 24 h with 7545 48 h, 8116 24 h against 8116 48 h and 7538 24 h and 7538 48 h. For ST comparison, analysis was performed by comparing the two isolates from within the same ST at the same timepoint. The ST101 24 h comparison consisted of isolates 7453 24 h and 8116 at 24 h, the ST101 48 h comparison was against 7453 48 h and 8116 48 h. The ST2 24 h analysis was between 7545 24 h and 7538 24h and the ST2 48 h comparison contained 7545 48 h and 7538 48 h. The draft genome sequences of 7538 and 8116 were used as the reference genome for ST2 and ST101, respectively. Functional annotation was performed with Eggnog mapper v2 (v2.0.0) using Listeriaceae as the taxonomic scope and gene ontology from experimental evidence only with all other fields default. The functional annotation was matched to differentially expressed genes (DEGs) using Excel and were analysed based upon their clusters of orthologous groups (COG) category with tRNAs allocated to COG category J and hypothetical proteins and DEGs with no COG category assigned to category S to include in the analysis. Overexpressed protein pathways were determined using STRING (v11) (29, 30) by submitting the amino acid sequences for all the DEGs (FDR <0.01 and log_2 FC) with *L. monocytogenes* EGD-e as the organism reference. Statistical significance was determined for overexpressed protein pathways with a false discovery rate (FDR) <0.01 and absolute log fold change (logFC) of ≥2 for 24 h vs 48 h samples. Differentially expressed regulatory genes were determined utilising an FDR <0.05. Regulatory genes of interest were determined based upon the conditions utilised in the model biofilm system and reflected the isolates potential systems/pathways used to respond to these conditions and included the following genes: *ctsR, hcrA, lexA, perR, codY, agrA, sigB, fur, recA, mogR, degU, virR* and *prfA*.

Results

Biofilm formation on SS coupons

Biofilm formation was assessed on SS coupons at 24, 48, 72 and 96 h. Isolates were examined based on mean biofilm cell density (log_{10} CFU/cm^2) with a broad distribution observed at each timepoint (Table 2) indicating all isolates were able to form biofilms.

Table 2. Mean biofilm density (log_{10} CFU/cm^2) range of all 52 *L. monocytogenes* biofilm isolates at sampled timepoints.

Time point	log_{10} CFU/cm^2	Variation in cell numbers (log_{10} CFU/cm^2)
24 h	1.20 - 4.16	2.95
48 h	2.61 - 5.39	2.78
72 h	4.51 - 5.83	1.32
96 h	4.40 - 5.82	1.41

Greatest differences in mean biofilm density between strains was observed at 24 and 48 h (Supplementary Figure 1; appendix A). A fast biofilm forming group with cell densities of 3.5 – 4.2 log_{10} CFU/cm^2 formed after 24 h was well separated from a group of slow biofilm forming isolates which only reached 1.2 – 1.8 log_{10} CFU/cm^2 after 24 h (Figure 1). This separation was less evident by 72 and 96 h with the mean cell densities being within 1.5 log_{10} when comparing isolates. The five isolates with the highest biofilm cell densities at 24 h were deemed to be the fast biofilm forming group (appended with ^{F} for clarity), which consisted of 7921^F, 7453^F, 7425^F, 7545^F and 7546^F. The five isolates with the lowest biofilm cell densities at 24 h were considered slow biofilm formers (appended with ^{S}) and included 7488^S, 8116^S, 7536^S, 7514^S and 7538^S; together, these fast and slow biofilm groupings make up the B10 isolates. At 24 h, three of the five isolates in the slow biofilm forming group were from Lineage I and four of the five isolates from the fast biofilm forming group were from Lineage II. Two of the isolates from

the fast biofilm forming group were from MLST ST155. MLST ST101 and ST2 had an isolate in both groups whereas all the other isolates in the two groups were from different STs.

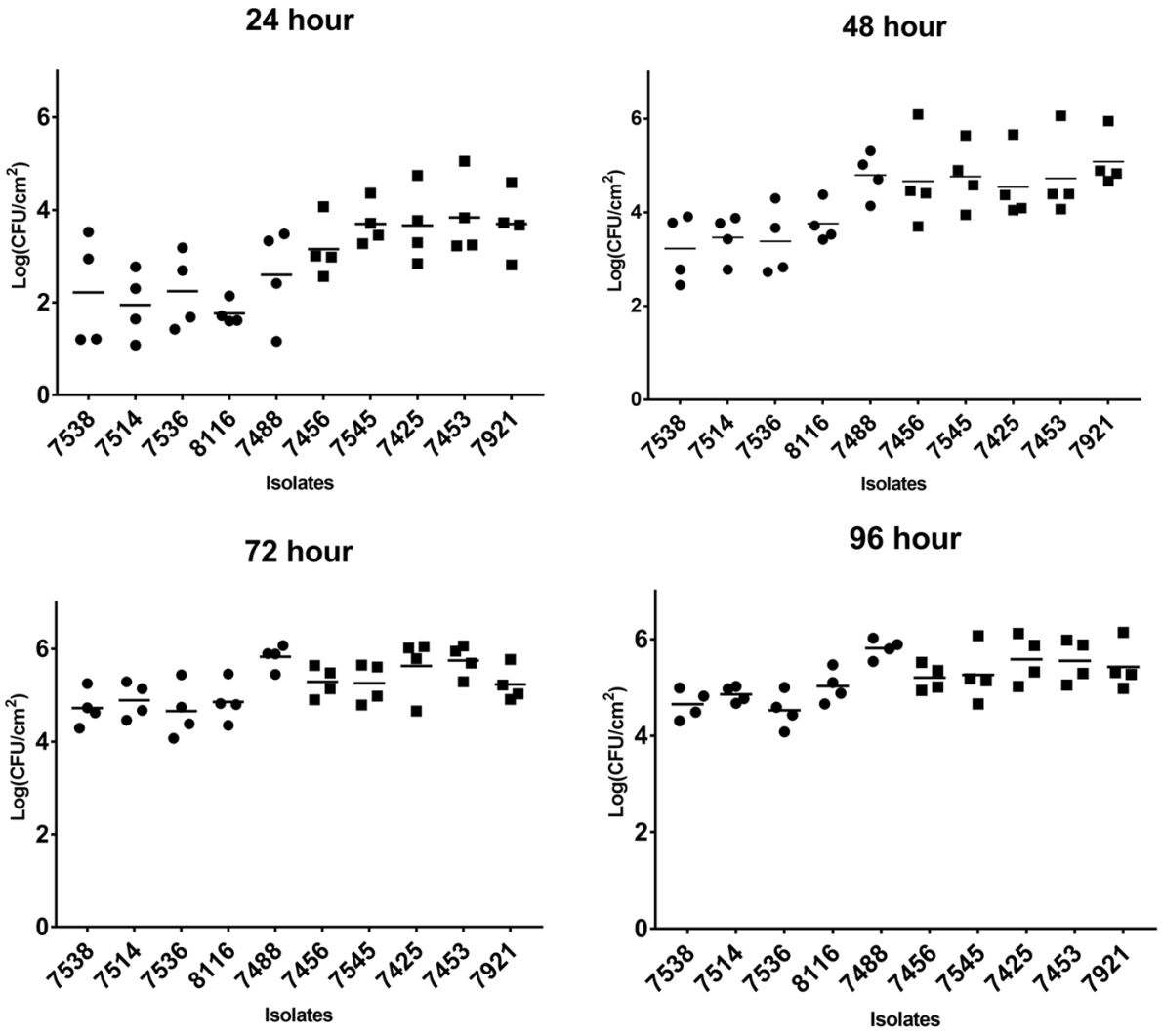

Figure 1. Comparison of the B10 isolates identified as displaying faster or slower biofilm formation over 96 h. Biofilm density (log_{10} CFU/cm^2) was determined every 24 h by standard plate count. Data points represent the average of four biological experiment replicates. •, slow isolates; ▪, fast isolates.

Genome wide association study

A microbial genome wide association study was performed across the 52 isolates utilising the biofilm phenotypic data to assess if there were any genetic differences associated with biofilm

formation and attachment ability. No significant single nucleotide polymorphisms (SNPs) associated with a faster or slower biofilm formation phenotype were identified amongst the 52 isolates; similarly, no genes showed statistically significant phenotypic association. Phylogenetic association was determined by treeWAS based upon 28,414 core SNPs resulting in isolates grouping by clonal complex (Supplementary Figure 2; appendix A).

EPS production

The ability to produce exopolymeric substances was assessed in the B10 group to investigate if these features influence the ability of isolates to attach and form biofilms faster. In this study, isolates which showed a pink phenotype at 14°C regardless of the growth media were 7425^{F}, 7453^{F}, 7488^{S}, 7514^{S}, 7536^{S} and 7538^{S} illustrating an intermediate ability to bind Congo red and thus produce some form of EPS (Supplementary Figure 3; appendix A). A translucent phenotype was displayed by isolates 7456^{F}, 7545^{F}, 7921^{F} and 8116^{S} indicating they were unable to bind the Congo red dye and therefore did not produce EPS. At 37°C most isolates displayed the same phenotype as they did at 14°C although some changed phenotype, such as isolates 7453^{F} and 7488^{S} which became translucent, and 7456^{F} which produced a pink phenotype. Isolates 7536^{S} and 7538^{S} changed from the pink phenotype to translucent at 37°C when grown in MHB. The above phenotypes were not associated with a slow or fast biofilm formation group.

Growth rate and doubling time of B10

The growth rate of the B10 isolates at 14°C in dBHI and at 37°C in BHI was determined (Figure 2). At 14°C the isolates growth rate ranged from 0.00060 to 0.00093 min^{-1}. The slowest growth rate was associated with isolate 7545^{F} with the fastest growth rate belonging to isolate 7488^{S}. The doubling time was also measured with a broad range of times observed (12.4 h to 19.9 h). At 37°C the growth rate and doubling times ranged from 0.01315 to 0.01468 min^{-1} and 43.55 min to 48.61 min, respectively, reflective of typical *L. monocytogenes* growth under

optimal conditions. Importantly, growth rate and doubling times were not correlated to biofilm forming ability at either temperature.

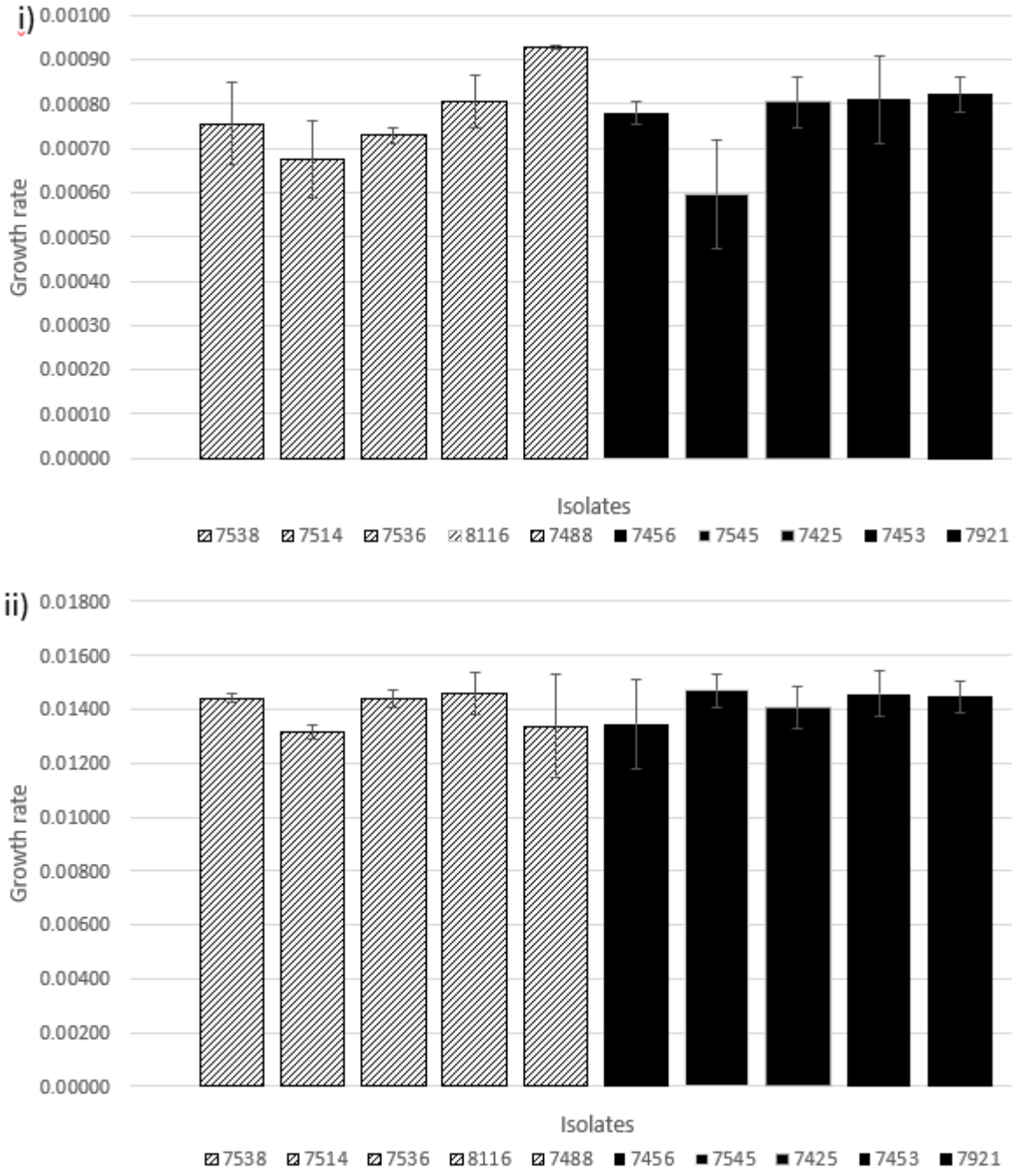

Figure 2. **Mean specific growth rate of the B10 isolates and standard deviation at: i) 14°C in dBHI (three biological replicates) and; ii) 37 °C in full BHI (two biological and seven technical replicates).** Patterned bars – slow isolates; solid black bars – fast isolates.

agrD gene expression

The expression levels of the propeptide *agrD* were assessed using qRTi-PCR in the B10 isolates on coupons and in SM at 24 and 48 h. The Wilcoxon rank sum test indicated there was no statistically significant difference in *agrD* expression when comparing the fast and slow isolates against the independent growth conditions and timepoints. When the *agrD* expression is assessed by either paired condition or timepoint some differences are determined (Figure 3). Notably, *agrD* expression was upregulated in the early stages of attachment and biofilm development, relative to other conditions tested.

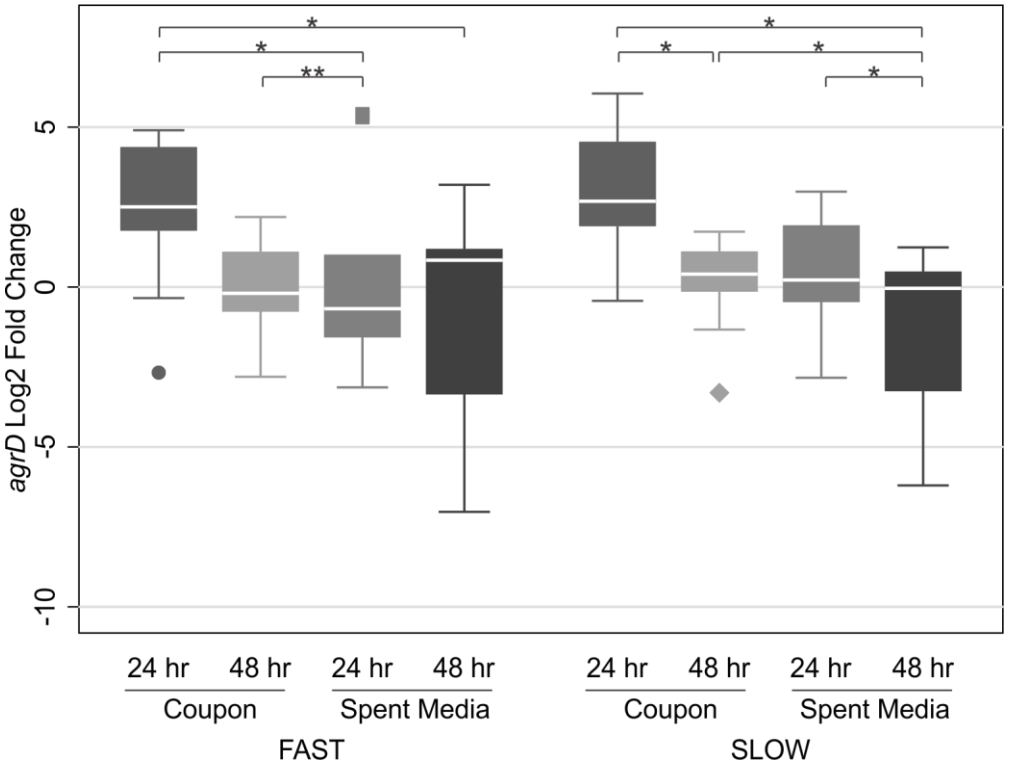

Figure 3. ***agrD*** **expression (log_2 fold change) at 24 and 48 hr in coupons (biofilm) and spent media (planktonic), normalised to *rpoB*.** No statistically significant difference in *agrD* expression between slow and fast isolates. Comparison of paired conditions or timepoints displayed significant difference, specifically C24hr Fast and SM24hr Fast (Z=2.073, P=0.0382), C48hr Slow and SM48hr Slow (Z=1.992, P=0.0464), C24hr Slow and C48hr Slow (Z=2.490, P=0.0128), SM24hr Slow and SM48 Slow (Z=2.192, P=0.0284), C24hr Fast and SM48hr Fast (Z=2.341, P=0.0192), C48hr Fast and SM24hr Fast (Z=2.970, P=0.0030) and C24hr Slow and SM48hr Slow (Z=2.521, P=0.0117). * p value <0.05; ** p value <0.01. C, coupon; SM, spent media; Z, z score, shading refers to the different experimental conditions.

Transcriptional analysis

Differentially expressed genes (DEGs) under food production environment biofilm formation conditions

The global transcriptomic changes in biofilm formation at 24 h and 48 h in dBHI was assessed for four individual isolates with two isolates from both the slow and fast biofilm formation groups, respectively (Supplementary Table 2; appendix A). The isolates chosen represented ST101 and ST2 with a fast and slow isolate in each ST. The number of reads ranged from 21,954,948 to 65,818,623 and were mapped to each isolate's individual genome. A total of 494 differentially expressed genes (DEGs) were identified using a false discovery rate (FDR) of < 0.01 and log fold change (logFC) of ≥2 across all comparisons. Isolate 7538^{S} and ST2 at both timepoints had no DEGs which met the FDR and log_2FC cut-off. At 24 h isolates 7453^{F}, 7545^{F} and 8116^{S} had 286, 76 and 7 DEGs respectively resulting in a total of 369 up regulated DEGs. At 48 h isolates 7453^{F} and 7545^{F} had 85 and 23 DEGs respectively totalling 108 DEGs. Between ST101 there was 11 up regulated and 6 down regulated DEGs at 24 h and 48 h, respectively. The DEGs were annotated in Eggnog.

Functional annotation of transcriptome

The clusters of orthologous groups (COGs) were used to identify the functional categories of the DEGs. The DEGs were allocated to 20 of the COG categories (Supplementary Table 3; appendix A) with 19 DEGs assigned to multiple (> 1) COG categories and were therefore treated as belonging to both COG categories. Approximately a third (30 %) of the DEGs were allocated to the 'S' COG categories 'Function unknown'. Amongst the 24 h up regulated DEGs with functional assignments the next three prevalent COG categories are 'G: Carbohydrate transport and metabolism', 'J: Translation, ribosomal structure and biogenesis', and 'M: Cell wall/membrane/envelope biogenesis.' The top three amongst 48 h up regulated DEGs also includes categories 'G' and 'J' along with 'K: Transcription.'

Pathways identified

The STRING database was used to identify overexpressed pathways and the molecular mode of action present within the DEGs of isolates 7453^F, 7545^F and ST101 at 24 and 48 h (Figure 4 and Table 3). The string analysis scores genes that are consistently and similarly expressed and are shown in association in Figure 4. In isolate 7453^F, the phosphotransferase system (PTS) (FC range 3.48 - 6.10) and starch and sucrose metabolism pathways (FC range 3.66 – 6.10) were overexpressed at 24 h along with cobalamin biosynthesis (FC range 3.35 – 5.05). The pathways for amino sugar and nucleotide sugar metabolism (FC range 2.99 – 3.89) were overexpressed at 48 h. The overexpressed pathway identified in isolate 7545^F at 24 h included starch and sucrose metabolism (FC range 2.87 – 3.69). At 48 h the pathway overexpressed was ribosome (FC range 3.36 – 4.56) associated with various RNA proteins and ribosomal domains identified. Pyrimidine metabolism and alanine, aspartate and glutamate metabolism (FC range – 4.20-4.37) were pathways down regulated for the ST101 48 h DEGs (Table 4). The upregulated DEGs of ST101 at 24 h predominately consisted of prophage genes (FC range 9.61 – 12.10). In addition, isolate 7453^F at 24 h also contain prophage up regulated DEGs (FC range 3.34 – 6.20). Most of the molecular action consisted of post translational modification, reaction, binding and catalysis.

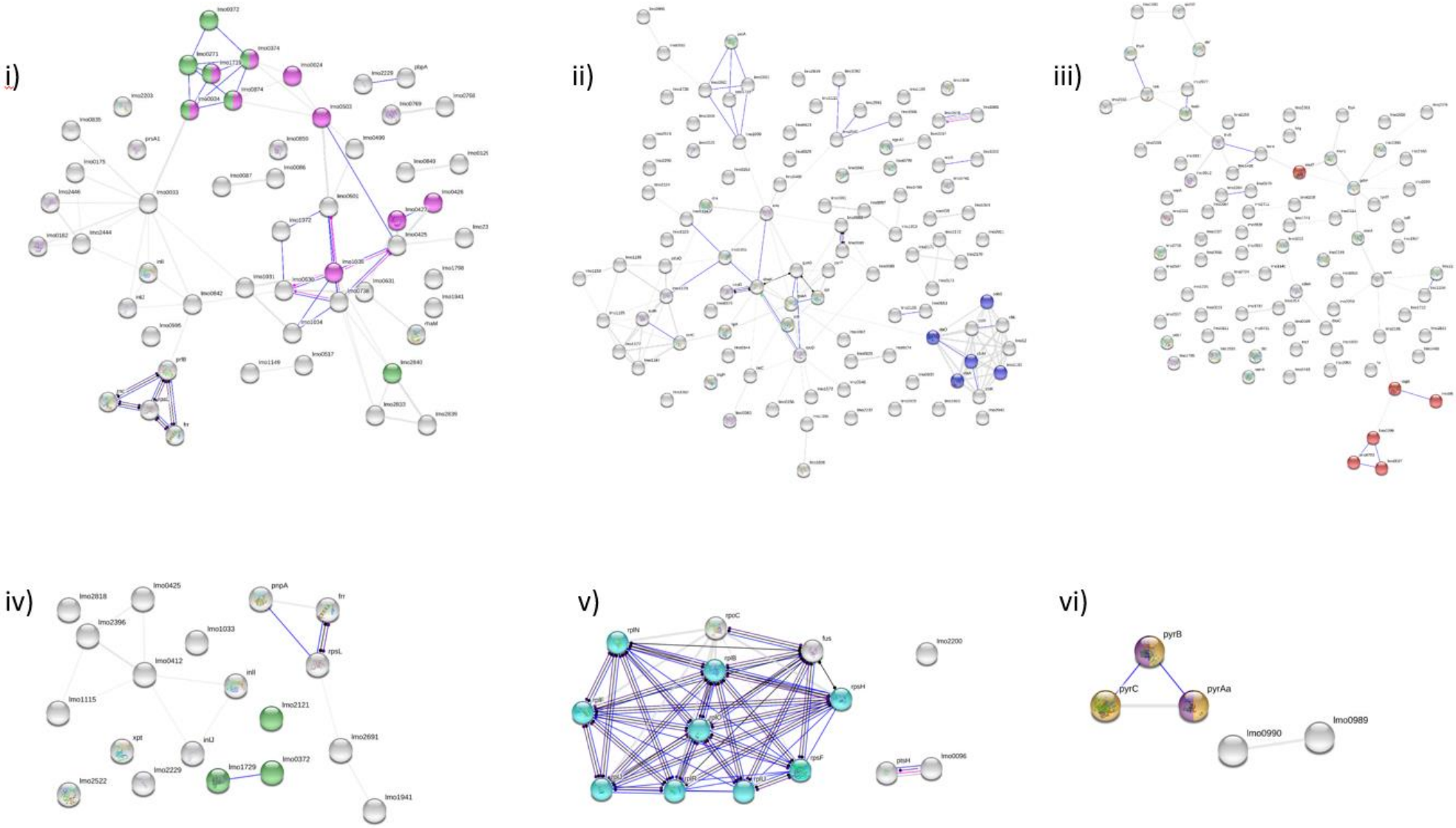
i)
ii)
iii)
iv)
v)
vi)
pyrB
pyrC
pyrAa
lmo0990
lmo0989

Figure 4. Overexpressed protein pathways in the transcriptome at 24 and 48 h in isolates 7453, 7545 and ST101 48 h. i & ii) 7453 24 h; iii) 7453 48 h; iv) 7545 24 h; v) 7545 48 h; vi) ST101 48 h. Coloured nodes relate to overexpressed pathways: pink, phosphotransferase system; light green, starch and sucrose metabolism; dark blue, cobalamin biosynthesis; red, amino sugar and nucleotide sugar metabolism; light blue, ribosome; yellow, pyrimidine metabolism; and purple, alanine, aspartate and glutamate metabolism. Coloured lines connecting nodes relate to action type: blue, binding; black, reaction; purple, catalysis; and pink, post-translation modification. Locus tags and genes names are based upon matches to proteins in the reference genome, *L. monocytogenes* EGD-e.

Table 3. Overexpressed pathways in differentially* expressed genes at 24 and 48 h in *L. monocytogenes* isolates 7453^{F} and 7545^{F}.

Locus tag (EGD-e)	Locus tag (this study)	Fold Change	Gene	COG cat	Description	KEGG Enzyme	Isolate & TP#
					PTS system		
lmo1035	fig|1639.4014.peg.1355	4.22		G	PEP-dependent sugar PTS, EIIA 1		7453
lmo1719	fig|1639.4014.peg.1473	4.36		G	PTS system cellobiose-specific IIA component	2.7.1.205	24 h
lmo0427	fig|1639.4014.peg.2115	4.77		G	PTS system, Lactose/Cellobiose specific IIB subunit		
lmo0426	fig|1639.4014.peg.2116	4.02		G	PEP-dependent sugar PTS, EIIA 2		
lmo0024	fig|1639.4014.peg.434	4.99		G	PTS system mannose/fructose/sorbose family IID component		
lmo0034	fig|1639.4014.peg.444	5.61		G	PTS system cellobiose-specific IIC component		
lmo0374	fig|1639.4014.peg.610	3.66		G	PTS system cellobiose-specific IIB component	2.7.1.205	
lmo0874	fig|1639.4014.peg.773	6.1		G	PTS system, Lactose/Cellobiose specific IIA subunit; PTS system beta-glucoside-specific IIA component		
lmo0503	fig|1639.4014.peg.936	3.48		G	PTS system galactitol-specific IIA component	2.7.1.200	
					Starch and Sucrose metabolism		
lmo0271	fig|1639.4014.peg.1428	4.47		G	Glycosyl hydrolase 1 family; 6-phospho-beta-glucosidase	3.2.1.86	7453
lmo1719	fig|1639.4014.peg.1473	4.36		G	PTS lichenan-specific enzyme IIA component; PTS system beta-glucoside-specific IIA component; PTS system cellobiose-specific IIA component	2.7.1.205	24 h
lmo2840	fig|1639.4014.peg.393	4.82	YcjM	G	Sucrose glucosyltransferase/sucrose phosphorylase (ycjM)	2.4.1.7	
lmo0034	fig|1639.4014.peg.444	5.61		G	PTS system cellobiose-specific IIC component		
lmo0372	fig|1639.4014.peg.608	4.16		G	Glycosyl hydrolase 1 family; 6-phospho-beta-glucosidase	3.2.1.86	
lmo0374	fig|1639.4014.peg.610	3.66		G	PTS system cellobiose-specific IIB component	2.7.1.205	
lmo0874	fig|1639.4014.peg.773	6.1		G	PTS system, Lactose/Cellobiose specific IIA subunit; PTS system beta-glucoside-specific IIA component		

lmo1729	fig|1639.4024.peg.1538	3.27		G	Glycosyl hydrolase 3 family; beta-glucosidase	3.2.1.21	7545
lmo0372	fig|1639.4024.peg.670	3.69		G	Glycosyl hydrolase 1 family; 6-phospho-beta-glucosidase	3.2.1.86	24 h
lmo2121	fig|1639.4024.peg.985	2.87		G	Trehalose and maltose hydrolases; Maltose phosphorylase	2.4.1.8	
					Cobalamin biosynthesis		
lmo1148	fig|1639.4014.peg.281	5.05	cobS	H	Cobalamin synthase	2.7.8.26	7453
lmo1192	fig|1639.4014.peg.237	3.51	cobD	H	Adenosylcobinamide-phosphate synthase	6.3.1.10	24 h
lmo1194	fig|1639.4014.peg.235	3.95	cbiD	H	Cobalt-precorrin-5B (C1)-methyltransferase	2.1.1.195	
lmo1191	fig|1639.4014.peg.238	3.35	cbiA	H	Cobyrinic acid c-diamide synthetase	6.3.5.11	
lmo1204	fig|1639.4014.peg.225	4.61	cbiM	P	Cobalt ECF transporter substrate-binding protein CbiM		
					Prophage related genes		
	fig|1639.4014.peg.1757	3.84		K	BRO family, N-terminal domain; Antirepressor [Bacteriophage A118]		7453
	fig|1639.4014.peg.1796	3.38		N	Bacterial Ig-like domain 2; Protein gp13 [Bacteriophage A118]		24 h
	fig|1639.4014.peg.2230	3.41		S	Phosphoadenosine phosphosulfate; Co-activator of prophage gene expression IbrA		
	fig|1639.4014.peg.2484	5.39		S	Phage protein		
	fig|1639.4014.peg.1804	4.2		S	Putative short tail fibre [Bacteriophage A118]		
	fig|1639.4014.peg.1780	4.16		S	Protein of unknown function (DUF2481) [Bacteriophage A118]		
	fig|1639.4014.peg.2780	4.01		S	Prophage endopeptidase tail		
	fig|1639.4014.peg.1788	3.89		S	Phage minor capsid protein 2		
	fig|1639.4014.peg.1793	3.87		S	Minor capsid protein		
	fig|1639.4014.peg.1805	3.8		S	Protein gp22 [Bacteriophage A118]		
	fig|1639.4014.peg.2062	3.61		S	Phage tail tape measure protein		
	fig|1639.4014.peg.2783	3.41		S	COG5546 Small integral membrane protein		
	fig|1639.4014.peg.1787	3.34		S	Phage portal protein, SPP1 Gp6-like [Bacteriophage A118]		
	fig|1639.4014.peg.1759	6.2		S	Protein gp44 [Bacteriophage A118]		

	fig|1639.4014.peg.468	5.43		V	Type VII secretion protein EsaA		
Amino sugar and nucleotide sugar metabolism							
lmo0957	fig|1639.4014.peg.2911	3.89	nagB	G	Glucosamine-6-phosphate deaminase	3.5.99.6	7453
lmo0956	fig|1639.4014.peg.2912	3.55	nagA	G	N-acetylglucosamine-6-phosphate deacetylase	3.5.1.25	48 h
lmo0096	fig|1639.4014.peg.505	3.03		G	PTS system mannost-specific transporter subunits IIAB	2.7.1.191	
lmo0097	fig|1639.4014.peg.506	2.99		G	PTS system mannose-specific IIC component		
Lmo0783	fig|1639.4014.peg.1297	4.18	manX	G	PTS system mannose-specific IIAB component	2.7.1.191	
lmo2552	fig|1639.4014.peg.192	3.7	murZ	M	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	2.5.1.7	
Ribosome							
lmo1542	fig|1639.4024.peg.1293	3.82	rplU	J	LSU ribosomal protein L21p		7545
lmo0250	fig|1639.4024.peg.1620	3.36	rplJ	J	LSU ribosomal protein L10p (P0)		48 h
lmo2629	fig|1639.4024.peg.266	4.02	rplB	J	LSU ribosomal protein L2p (L8e)		
lmo2622	fig|1639.4024.peg.273	3.85	rplN	J	LSU ribosomal protein L14p (L23e)		
lmo2618	fig|1639.4024.peg.277	4.56	rpsH	J	SSU ribosomal protein S8p (S15Ae)		
lmo2617	fig|1639.4024.peg.278	4.01	rplF	J	LSU ribosomal protein L6p (L9e)		
lmo2616	fig|1639.4024.peg.279	3.85	rplR	J	LSU ribosomal protein L18p (L5e)		
lmo2613	fig|1639.4024.peg.282	3.57	rplO	J	LSU ribosomal protein L15p (L27Ae)		
lmo0044	fig|1639.4024.peg.808	4.46	rpsF	J	SSU ribosomal protein S6p		

* FDR <0.01 $\log_2$ fold change

Time point

Table 4. ST101 pathways overexpressed in differentially* expressed genes at 24 and 48 h in *L. monocytogenes*.

Locus tag (EGD-e)	Locus tag (this study)	FC	Gene	COG Cat	Gene/Protein Name	KEGG	Isolate & TP#
				Prophage related genes			
	fig|1639.4037.peg.3124	9.61		S	Microvirus J protein; Phage DNA binding protein		ST101 24 h
	fig|1639.4037.peg.3125	10.88		S	Bacteriophage scaffolding protein D		up
	fig|1639.4037.peg.3120	10.95		S	Bacteriophage replication gene A protein (GPA)		regulated
	fig|1639.4037.peg.3126	10.99		S	Phage protein C; Phage single stranded DNA synthesis		
	fig|1639.4037.peg.3123	11.06		S	Capsid protein (F protein); Phage major capsid protein		
	fig|1639.4037.peg.3121	11.21		S	Microvirus H protein (pilot protein); Phage minor capsid protein		
	fig|1639.4037.peg.3122	11.83		S	Major spike protein (G protein)		
	fig|1639.4037.peg.3127	12.1		S	Bacteriophage replication gene A protein (GPA)		
				Pyrimidine metabolism and Alanine, aspartate and glutamate metabolism			
lmo1838	fig|1639.4037.peg.1939	-4.37	pyrB	F	Aspartate carbamoyltransferase	2.1.3.2	ST101 48 h
lmo1837	fig|1639.4037.peg.1938	-4.32	pyrC	F	Dihydroorotase	3.5.2.3	down
lmo1036	fig|1639.4037.peg.1937	-4.2	pyrAa	F	Carbamoyl-phosphate synthase small chain	6.3.5.5	regulated

* FDR <0.01 $\log_2$ fold change

Time point

Differential expression of select regulator genes

Seven of the regulatory genes selected for their association with stress response were significantly differentially expressed (DE) across three of the isolates (Table 5). Three regulatory genes, *fur*, *lexA* and *recA* were DE in isolate 7453 at 48 h with the logFC range between 2.56 and 4.16. Isolate 7538 displayed DE of four genes, *ctsR*, *degU*, *recA* and *sigB* at 48 h with the logFC ranging from 3.22 to 3.81. The *mogR* gene (logFC 1.67) and *recA* (logFC 2.32) gene were DE in isolate 7545 at 24 h and 48 h, respectively. All other time points and isolates were negative for significant differential expression of the selected regulatory genes. Interestingly, *recA* was the only regulatory gene which was DE across three different isolates all at the 48 h timepoint and a logFC range from 2.32 to 3.42.

Table 5. Fold change of regulator genes differentially expressed at FDR <0.05.

	7453^{F}		7538^{S}		7545^{F}	
	24 h	48 h	24 h	48 h	24 h	48 h
ctsR				3.61		
degU				3.22		
fur		4.16				
lexA		2.82				
mogR					1.67	
recA		2.56		3.42		2.32
sigB				3.81		

Discussion

Listeria monocytogenes' ability to colonise the FPE is a concern for the health of the at-risk population and the processing facilities' economic viability and reputation. A deeper knowledge of *L. monocytogenes'* ability to colonise and survive in the FPE is required. The ability to replicate conditions representative of the FPE will assist in improving our understanding of these dynamics, however there are multiple complex elements involved including the type of contact surfaces (food and non-food) present, temperature, time,

nutrients and ability to form biofilms. The availability and type of nutrients varies depending on the type of food or food products processed. While it is difficult to replicate the exact nutritional content available in the FPE, it is known to alternate between high and low nutritional stages during cycles of production. In this study, we assessed colonization behaviour of *L. monocytogenes*, incorporating these factors to reflect those of the FPE.

Initially there was some debate in the literature on *L. monocytogenes* ability to form biofilms, however there is growing evidence to suggest biofilm formation is a key component of the survival and persistence of some strains (16, 33-35). Early studies have tried to associate biofilm formation to a lineage or serotype with varying results (36-38). In this study, biofilm formation was observed to be strain-specific as there was no consistency in the fast or slow biofilm groups linking a given phenotype to a specific genotype, as discussed below, and initially suggested in earlier *L. monocytogenes* biofilm studies (38-41). Although two isolates belonging to MLST ST155 were present in the faster biofilm forming group, ST101 and ST2 had an isolate in both faster and slower groups, indicating no clear phenotype association with genetic sub-lineage. As attachment and biofilm formation appears to be environment and strain-specific we sought to determine what additional components may be of influence.

In the FPE, access to nutrients can be transient therefore *L. monocytogenes* cells need to be able to adapt to the environmental conditions available. Biofilm formation studies have assessed the impact of nutrient deprivation, such as the study by Kadam et al (38) reporting enhanced biofilm formation and attachment was positively influenced in nutrient poor media. Cherifi et al (42) assessed BHI and a diluted BHI media with similar results. The results from this study correlate with *L. monocytogenes* ability to form biofilms in a low nutrient environment. This ability to adapt to low nutrient conditions may account for some of the differences in biofilm formation seen at 24 h, however by 96 h these variances were not apparent; this was also observed by Harvey et al (43), indicating initial attachment within 24-48 h is key to FPE colonisation.

A potential influence on attachment and biofilm formation during the first 24 h is the growth rate of isolates. While it is well known there can be differences in growth rate between strains, in this study the ability to form biofilms was not associated with growth rate and doubling times at 14 or 37°C which reflects the results of other published research. The independent nature of biofilm formation to growth rate has been reported in previous studies at temperatures reflecting FPE and also at 37°C (37, 44). Lee et al (45) noted less biomass was produced at 10°C compared to biofilms at 37°C, which were attributed to a lower growth rate and cell hydrophobicity at the cold temperature. Taylor and Stasiewicz (46) also found persistent strains did not display increased ability to grow in various energy sources and conditions with their ability to persist most likely strain-specific or the result of environmental conditions.

The extracellular matrix (ECM) is a necessary component of the biofilm structure and is composed of proteins, extracellular DNA, polysaccharides and exopolysaccharides and amyloid fibres, however the composition varies between species (47). While *Listeria* is not known to be a producer of cellulose, curli fimbriae and poly-β-1,6-N-acetyl-D-glucosamine common amongst Proteobacteria which produce defined biofilms, it has been reported *Listeria* produces a novel EPS primarily composed of N-acetylmannosamine and galactose which is capable of binding congo red as an indicator (48, 49). Two phenotypes were present in the B10 group, pink indicative of some EPS production and translucent, negative for EPS production, with the production depending on the medium used for some strains. While the amount of EPS produced was not determined, the presence of EPS in *Listeria* has been linked with cell aggregation and increased tolerance to disinfectants and desiccation suggesting the B10 strains which are capable of producing EPS have increased ability to survive and persist within the FPE and display initial stages of biofilm formation (48). EPS production was not associated exclusively with either faster or slower biofilm formation.

The *agr* system was initially described as a signalling peptide system in staphylococcal species (50), with orthologs *lam* (51) and *fsr* (52) being identified in *Lactobacillus plantarum* and *Enterococcus faecalis*, respectively, in addition to *L. monocytogenes*. The *agr* system is a peptide signalling communication four gene operon composed of *agrB*, a transmembrane protein which processes the propeptide encoded by *agrD* into a mature autoinducing peptide (AIP). The AIP is then exported into the extracellular environment until the concentration achieves a certain threshold, triggering the histidine kinase sensor *agrC* and activating the response regulator *agrA* which combine as a two-component system (*agrC-agrA*) applying transcriptional regulation including positive regulation (53-57). The *agr* system has been shown in *L. monocytogenes* to be involved in invasion, pathogenicity and biofilm formation (58). While this system has been shown to be linked to biofilm formation there is limited research on differences in expression between strong and poor biofilm producers at conditions reflecting the FPE. In this study, there was some statistical differences when comparing cells isolated from coupons to SM within either the fast or slow group; however, there was no statistical difference in the expression of *agrD* between the fast isolates and the slow isolates. Gandra et al (59) reported higher levels of the *agr* locus is expressed at 37°C compared to 10°C. In addition, they identified *agrBCD* genes are important for adhesion and the initial stages of biofilm formation particularly at 12 and 24 h. The results of this study support the upregulation of *agr* system elements in the early stages of attachment and early biofilm growth; however, expression appears to decline as the biofilm matures. In contrast, increased *agrD* expression was not observed in the planktonic cells of the spent media in this study at any of the timepoints measured, suggesting expression of this signal peptide is induced following attachment and initial biofilm formation, rather than planktonic growth, under the conditions tested.

To further investigate a genetic basis for the rapid colonisation phenotype, this study also examined the global transcriptomic response of *L. monocytogenes* during attachment and biofilm formation at 24 and 48 h under conditions reflective of the FPE. Four isolates from two

STs (two isolates per ST) were chosen for RNA sequencing, with each ST cohort including one fast and one slow coloniser, to provide insights into variation in gene expression between fast and slow colonisation phenotypes. This included a lineage I and lineage II ST. Globally across strains metabolism and transport pathways were up regulated with variation of the pathways between strains. As a saprophyte, *L. monocytogenes* is exposed to varied, and at times limited nutrient sources and as such requires an extensive range of transport and metabolism mechanisms. Glaser et al (60) identified 331 different transporter genes with 88 related to the phosphoenolpyruvate-dependent phosphotransferase systems (PTS) responsible for the transport and phosphorylation of various sugars and sugar derivates (61). This extensive range of transporter genes is one of the largest known among bacterial species and allows *L. monocytogenes* to survive within a broad range of environmental and host conditions (60). Furthermore, it allows for the bacterium to respond to any changes in its environment and adapt, as necessary. In a few other bacterial species in which the biofilm genetic landscape has been eluded, PTS has been linked with the regulation of biofilm formation. In a study on *Klebsiella pneumoniae* biofilms, three genes encoding an enzyme II complex in PTS was found to increase eDNA and capsular polysaccharide production resulting in positive regulation of biofilm production (62). Similarly, Houot and Watnick (63) found the *Vibrio* polysaccharide (*vps*) genes of *Vibrio cholerae* responsible for exopolysaccharide synthesis, were coregulated with PTS components and formation of multilayer biofilms were influenced by particular PTS sugars which activated the transcription of these *vps* genes. Unlike *V. cholerae*, the genetic determinants for *L. monocytogenes* biofilms are not well defined and comprise of a variety of genetic interactions, with most also having an established role in virulence and pathogenicity. In our study, various components of the PTS were upregulated at 24 h across the fast isolates, compared to the slow isolates, however there is limited research assessing how the PTS influences biofilm formation at conditions reflective of the FPE in *L. monocytogenes*. In this study, various elements of the PTS pathways up regulated in different strains further suggests colonisation differences are strain-specific and influenced by environmental conditions. Further research is required to

determine if various components of the PTS are responding to their preferred nutrients as the result of the isolation environments selected in this study, or if the PTS has roles in the early stages of biofilm formation.

In conjunction with the PTS, various metabolic pathways associated with carbohydrates and sugars were also upregulated, including starch and sucrose metabolism at 24 h and amino and nucleotide sugar metabolism at 48 h across the fast isolates suggesting a switch to nutrient scavenging to initiate colonisation. Free glucose is often not readily available in the environment and as such alternative carbon sources are required. As mentioned previously, *L. monocytogenes* has an extensive transport system allowing this bacterium the ability to utilise various environmental carbon sources at times when nutrients are limited. Energy sources like cellobiose, lactose, lichenan, trehalose, maltose and their associated degradation products were all up regulated in this study as well as the 6-phospho-β-glucosidase, which suggests beta-glucosides are used by these strains. Taylor and Stasiewicz (46) found 97 % of *L. monocytogenes* isolates tested (n=95) grew in defined media supplemented with cellobiose, fructose or glucose however, lactose and sucrose were unable to support the growth of 79 and 72 % of the isolates, respectively. An earlier study also reported fructose, mannose, cellobiose and trehalose were capable of supporting *L. monocytogenes* growth in the absence of glucose (64). Mannose and trehalose supplementation has also been shown to increase biofilm development over 12 days (65). The results of this study suggest a global upregulation of diverse metabolic pathways under nutrient limited, low temperature conditions may facilitate adaptation and maximised nutrient scavenging, contributing to initiation of a biofilm lifestyle and persistence of *L. monocytogenes* under similar conditions founds in FPE.

Amino sugar metabolism has been connected to energy production and biosynthesis of cell wall peptidoglycan and teichoic acids (66, 67). Key enzymes of the amino sugar and nucleotide sugar pathway up regulated in this study at 48 h include N-acetylglucosamine-6-phosphate deacetylase (*nagA*) and glucosamine-6-phosphate deaminase (*nagB*), indicating

at 48 h under conditions reflective of the FPE the fast isolates are undergoing an increase in biomass through the biosynthesis of peptidoglycan cementing their ability to survive in the FPE. N-Acetylglucosamine (GlcNAc) is an abundant carbon and nitrogen source found throughout the environment (as a chitin monomer) and as part of bacterial cell wall peptidoglycan (68); it has been reported *L. monocytogenes* can turnover between 30-50 % of its cell wall peptidoglycan every generation (66). The deacetylation of N-acetyl-glucosamine-6-phosphate by NagA into glucosamine-6-phosphate and acetate is a part of peptidoglycan degradation and thus cell wall recycling (66). Glucosamine-6-phosphate can be further transformed into fructose-6-P by NagB for energy production through the glycolysis pathway (67). An additional key enzyme in peptidoglycan biosynthesis is UDP–*N*-acetylglucosamine (UDP-GlcNAc) 1-carboxyvinyltransferase (MurA) responsible for the addition of enolpyruvyl from phosphoenolpyruvate to UDP-GlcNAc (69). The paralogue version, *murZ* was up regulated in this study. The combination of *nagA*, *nagB* and *murZ* suggests cells were possibly undergoing cell wall synthesis to increase biofilm mass. This adaptation again suggests a global switch to nutrient scavenging and biomass increase is a central strategy to the initial colonisation of the FPE by *L. monocytogenes*.

Three genes involved in pyrimidine metabolism and alanine, aspartate and glutamate metabolism pathways were upregulated at 48 h in the ST101 comparison. The genes observed related to pyrimidine metabolism are involved in *de novo* synthesis of uridine-monophosphate (UMP) starting from glutamine and include, *pyrAa*, carbamoyl-phosphate synthase small chain, glutamine-utilizing subunit of carbamoyl-phosphate synthetase, similar to the *carA* of the same role in *E. coli*, *pyrB*, catalytic subunit of aspartate carbamoyltransferase and *pyrC*, dihydroorotase (70, 71). A study by Pisithkul (72) into biofilm development of *Bacillus subtilis* found expression of pyrimidine synthesis enzymes and other nucleotides and biosynthetic precursors peaked at 16 h then declined slowly for the remainder of the study. In another study, Hingston (73) identified *pyrAaBC* genes were up regulated at 4°C during the transition to stationary phase. De novo synthesis of UMP has been linked to

biofilm formation and production of cellulose and curli fimbriae in *E. coli* through transcription of the *csgDEFG* operon (74). While the *pyr* operon has not been linked to biofilm formation in *L. monocytogenes*, our results suggest it may be linked in some way, however further research is required.

Interestingly, the cobalamin biosynthesis pathway and genes involved in the cobalamin-dependent gene cluster (CDGC) were also identified as being overexpressed at 24 h in isolate 7453^{F}. Cobalamin genes are responsible for vitamin B_{12} biosynthesis which is required as enzyme cofactors for various metabolic processes particularly during the metabolism of ethanolamine and 1,2-propanediol as carbon and nitrogen energy sources (75). Cobalamin biosynthesis can occur during aerobic respiration with *cob* genes or during anaerobic respiration utilising *cbi* genes (76). In this study, more *cbi* genes (compared to *cob*) from the Cobalamin anaerobic pathway were upregulated, in addition, genes involved in ethanolamine (FC range 3.2 – 6.8) and propanediol utilisation (FC range 2.03 – 4.2) (Supplementary Table 2) were also up regulated and have been shown to be activated during stressful, competitive conditions and during cold temperatures (77-80). In a transcriptomic study by Hingston and colleagues (73), they reported an increase in genes associated with ethanolamine utilisation at multiple growth phases at 4°C. The upregulation of the genes from the CDGC may reflect *L. monocytogenes* is experiencing stress as a result of the low temperature and limited nutrients within the biofilm state. These systems facilitate greater flexibility in nutrient scavenging and utilisation through metabolism of alternative substrates, which is critical for survival when optimal nutrients are unavailable or competition with other microbial species is ongoing (79, 81).

Ribosomes are essential protein synthesising components that are involved in sensing and responding to their environmental conditions (82). In prokaryotes they are composed of a 50S large subunit, where the peptide bonds are formed, and a 30S small subunit that binds the messenger RNA, creating a 70S ribosome (83). In this study, a variety of ribosomal proteins

were upregulated with a majority being the large subunit. Each subunit contains 30 and 20 ribosomal proteins (R-proteins) designated L or S for the 50S or 30S subunits, respectively. R-proteins have various roles including translation, assembly, cell proliferation and cellular differentiation with some of these roles essential for survival (84). In this study, up regulation of ribosome proteins may reflect the global level of transcription and translation is higher under conditions reflective of the FPE due to multiple sub-optimal factors at play, however, there is limited research on the R-proteins in *L. monocytogenes* to be able to elude to more specific roles in this study.

Within isolate 7453^{F} and ST101 at 24 h there was a considerable number of differentially expressed prophage genes expressed suggesting prophage genes may influence the initial stages of colonisation. Over 500 *L. monocytogenes* bacteriophages have been identified, with a large portion being temperate phages capable of inserting themselves into the bacterial chromosome (85). Temperate phages have been linked with providing increased fitness to host bacterial strains (86). A common temperate listeria phage A118 has been shown to insert itself into the competence protein K (*comK*). A study by Verghese et al (87) showed meat and poultry isolates containing the *comK* prophage were capable of growing to higher cell densities with the authors suggesting its insertion allows strains to adapt to niches which influence their colonisation and persistence in the FPE. In an earlier study on *E. coli* K12 strains containing cryptic prophage, they found increased fitness against osmotic, oxidative and acidic stress and increases in biofilm formation and growth (86). While there have been limited studies reporting lab based phenotypic benefits of *L. monocytogenes* isolates containing prophages, the up regulation of prophage genes in this study opens the possibility they may play some role in either low nutrient adaption, attachment or biofilm formation. In this study, phage A118 is inserted into the *comK* gene of isolate 7453^{F} suggesting the presence of phage A118 may influence this isolate's ability to rapidly colonise the FPE by increasing cell density and withstanding the suboptimal conditions found in FPE.

The DE of regulators and repressors involved in stress response and biofilm formation can be indicators of which stress systems are responding to sub-optimal conditions, it is important however to note that it is not one particular regulator being induced rather a variety of different regulators and repressors indicating the complex nature of the FPE and the overlap in stress response and virulence related genes and systems. In this study, *recA/lexA*, responsible for DNA repair and activation of the SOS response during stressful conditions in *L. monocytogenes*, was upregulated in three strains and one strain, respectively. The SOS response is required for bacterial adaptation, diversification and pathogenesis in a majority of species and has been reported to be required for biofilm formation in *E. coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Mycobacterium tuberculosis* (88). Van der Veen et al (89) showed *recA* also influences genetic variability through mutagenic repair during continuous flow biofilms. The mutagenic repair of DNA may be critical for biofilm formation and resistance to stress conditions along with the development of disinfectants and antibiotic resistance (90). The upregulation of *recA* and *lexA* is indicative of the stress conditions experienced in this study from the low temperature and limited nutrients utilised.

The presence of flagella and motility related genes have been shown to be involved in initial attachment stages and subsequent biofilm formation and colonisation in *L. monocytogenes* on various processing environment and produce surfaces (91, 92). In isolate 7538 the *degU* response regulator was upregulated. Previously, *degU* has been associated with flagella biosynthesis, chemotaxis, attachment and biofilm formation (93, 94). Gueriri et al (94) suggests that *degU* may play a role in biofilm formation that is distinct from the essential role it plays in regulating flagella synthesis. In addition, Pieta et al (95) showed *degU* was equally or significantly increased at 7°C when compared with 37°C. Therefore, the upregulation of *degU* is suggestive of cells undergoing biofilm formation, as strain 7538 was a slow biofilm former it may be the motility of cells at lower temperatures may be regulated later compared to fast biofilm formers.

The *mogR* gene is the transcriptional repressor of flagella motility at all temperatures and specifically at temperatures relevant for infection (96). Cordero et al (97) reported strains which demonstrated faster growth rates at low temperatures displayed reduced flagella expression to conserve energy yet remain prolific. Isolate 7545 displayed *mogR* expression suggesting flagella motility was reduced potentially as a metabolic function to save energy and continue multiplying in the limited nutrients and low temperature conditions used in this study.

The class three stress gene repressor, *ctsR*, which regulates class three heat shock genes was upregulated in isolate 7538. In addition, *ctsR* has also been indirectly linked with virulence, motility gene expression and has been shown to be coregulated with other regulators including *sigB*, *sigH*, *hrcA* and *prfA* (73, 98-100). The general stress response gene, *sigB*, was also upregulated in this isolate. The alternative sigma factor β (*sigB*) is a major stress response regulator of general stress response and class II stress genes which are required for various stress related conditions, including cold, acidic, osmotic, oxidative stress and high-pressure processing (101, 102). *SigB* has been shown to be required for the biofilm mode of life in both, static and continuous flow biofilms (101), in addition there are reports it is required during starvation survival in low nutrient environments (103). In this study, the suboptimal conditions used were to reflect the stressful climate in the FPE therefore the upregulation of *ctsR* and *sigB* is an adaptive mechanism *L. monocytogenes* most likely employees to survive within the FPE.

The *fur* gene is required to regulate intracellular levels of iron which is an essential cofactor required for many important enzymatic roles in bacterial cells (104, 105). *Fur* regulation has been linked with oxidative stress response and protection against ROS damage (106, 107). In addition, in low iron environments f*ur* regulation plays a significant role in sequestering iron within increased levels of *fur* transcription reported in these environments (106, 108). The upregulation of f*ur* may be indicative of low iron levels as a result of the limited nutrient environment or cold stress conditions utilised in this study. Further, *fur* regulation has been

linked with metabolic function in bacteria (108); the conditions in this study resulted in a diverse range of metabolic systems upregulated and therefore the upregulation of *fur* may be reflective of the metabolic state cells in biofilm are undergoing.

In this study we aimed to replicate elements present in the FPE to determine their influence on the colonisation by *L. monocytogenes*. Although the results obtained provide beneficial insight into our understanding of this subject, it was not without its drawbacks. The multiple factors analysed in combination have provided some generalised understanding and identified baseline research against more isolates is required. The congo red assay is an indicator of curli fimbriae and cellulose synthesis neither of which is produced by *L. monocytogenes* and therefore we can only hypothesis the pink staining of isolates is indicative of EPS production with further research into which components congo red is binding to required. For the ST comparison the isolates were not isogenic strains however based upon the average nucleotide identity (99.91 % for isolates 7453 and 8116 and 99.90 % for isolates 7545 and 7538) the isolates selected were considered suitable for comparison purposes. In addition, the expression data for a small number of genes which are not shared between the comparison isolates may be absent as a result of using non-isogenic strains.

Concluding remarks

L. monocytogenes isolates are a concern for public health due to their ability to colonise and persist in FPEs. The economic and brand reputation for a food processing company can be substantial should *L. monocytogenes* strains contaminate RTE food products and cause listeriosis. This study looked at various factors which may influence *L. monocytogenes* ability to colonise a processing facility. We demonstrated that the ability to form biofilms was different from strain to strain and was not linked to differences in growth at conditions reflective of the FPE, nor cellulose or curli expression as identified in other species like *E. coli* and *Salmonella*. While there were also no specific genes identified by the GWAS, interestingly the global

transcriptome indicated metabolic mechanisms were upregulated, suggesting the species utilizes its wide metabolic and transport repertoire to initiate a rapid adaptation to nutrient limited conditions. This is then coupled with upregulation of genes involved in the production of cell structural components for biofilm expansion, with upregulation of the *agr* system in the initial attachment and biofilm growth. Colonisation is likely aided through environmental factors like hard to clean and sanitise niches, and genetic determinants like the ability to form biofilms and attach in suboptimal conditions. Our knowledge of *L. monocytogenes'* ability to persist and survive in the FPE requires further exploration, as this knowledge will be necessary in order to prevent and mitigate contamination.

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CHAPTER 4

Characterisation of *Listeria monocytogenes* food-associated isolates to assess environmental fitness and virulence potential.

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Abstract

The ability of *L. monocytogenes* isolates to survive within the food production environment (FPE), as well as virulence, varies greatly between strains. There are specific genetic determinants that have been identified which can strongly influence a strains ability to survive in either domain. In this study, we assessed the FPE fitness, virulence and efficacy of hygiene or treatment intervention, against 52 *L. monocytogenes* strains isolated from various food and food environment sources. Phenotypic tests were performed to determine the minimum inhibitory concentration of cadmium chloride and benzalkonium chloride and the sensitivities to five clinically relevant antibiotics. A genomic analysis was also performed to identify resistance genes correlating to the observed phenotypic resistance profiles, along with genetic determinants of interest which may elude to the FPE fitness and virulence potential. A transposon element containing a novel cadmium resistance gene, *cadA7*, a Tn*916* variant insert in the hypervariable *Listeria* genomic island 1 region and an LGI2 variant were identified. Resistance to cadmium and disinfectants was prevalent among isolates in this study, although no resistance to clinically important antimicrobials was observed. Potential hypervirulent strains containing full length *inlA*, LIPI-1 and LIPI-3 were also identified in this study. Cumulatively, the results of this study show a vast array of FPE survival and pathogenicity potential among food production-associated isolates, which may be of concern for food

processing operators and clinicians regarding *L. monocytogenes* strains colonising and persisting within the FPE, and subsequently contaminating food products then causing disease in at risk population groups.

Introduction

Listeria monocytogenes is a saprophyte and a human pathogen. *L. monocytogenes* can cause the severe disease, listeriosis, in at risk populations that includes the elderly, immuno-compromised, pregnant women and neonates with an associated mortality rate of almost 30 % (1, 2). *L. monocytogenes* can enter the food production environment (FPE) through the introduction of raw ingredients, which can progress to the colonisation of the production environment and subsequent cross contamination of food products, particularly ready to eat (RTE) items (3). Although not all strains which enter the FPE will go on to cause listeriosis, there are a variety of genetic and phenotypic traits/mechanisms which can indicate a strains ability to survive or cause disease. An understanding of the various factors influencing colonisation, survival and pathogenicity is thus important.

Within the FPE there are intrinsic (food-related) and extrinsic (intentionally applied to reduce microbial contamination and spoilage) stress factors utilised to control *L. monocytogenes* strains including high osmolarity, temperature and pH, disinfectants, sanitisers and episodes of desiccation (4). *L. monocytogenes*' ability to survive various processing/hurdle technologies influences its ability to colonise and persist in the FPE, making it an important food borne pathogen. Survival throughout the FPE can result in cross contamination of products like RTE foods, which are considered high risk products due to the lack of further cooking prior to consumption, and common vehicles for listeriosis outbreaks (3, 4). Importantly, it has been suggested conditions present within the FPE may promote the development of survival strategies like cross protection and interconnectedness between tolerance or resistance to multiple stressors (5). At refrigeration temperatures, or lower ambient temperatures used in

many FPEs, an increase in tolerance of associated *L. monocytogenes* strains to cold temperatures, osmotic and oxidative stressors has been noted (6, 7). Recently identified genomic islands, stress survival islet (SSI)-1, and SSI-2, are responsible for tolerance to acid, salt, bile, gastric, alkaline and/or oxidative stress, further highlighting the diverse genomic arsenal which supports niche adaptation, survival and persistence of *L. monocytogenes* in the FPE (8, 9). In addition, the presence of plasmids and prophage elements also increases an isolates fitness within the FPE (10, 11).

An understanding of the pathogenic potential of *L. monocytogenes* strains isolated from the FPE, as well as their resistance to antibiotics, is also important. The listerial infection cycle is the result of several essential virulence factors, predominately a six gene virulence cluster also known as *Listeria* pathogenic island 1 (LIPI-1) (12-16). In addition, there are other important genes involved in virulence, including *inlA* and *inlB*, which are required for initial invasion (17). However, not all isolates in the FPE contain functional virulence genes, with mutations in key virulence genes like *prfA* or *inlA* resulting in a reduced pathogenic potential (18, 19). Determining the presence and the degree of diversity can provide an overview of the pathogenicity potential of transient or persistent isolates within the FPE (16). Differences in virulence is an important public health concern as highly virulent strains can be associated with outbreaks and severity of illness, and therefore the sensitivity to clinically relevant antibiotics is also required. Importantly, resistance in *L. monocytogenes* isolates has been increasingly reported, along with the presence of genetic determinants being identified for various classes of antibiotics, some of clinical relevance (20-22); therefore, continued surveillance of antibiotic sensitivity is required. Typically, the pathogenic potential of clinical isolates is looked at, with less studies considering food-isolated *L. monocytogenes* ability to both cause disease, in addition to the carriage of genes that may support their survival within the FPE. The aim of this study was to characterise *L. monocytogenes* isolates from food and food-environment sources using phenotypic and genomic methods to determine their ability

to survive within the food production environment, their potential to cause infection and their susceptibility to frequently used antibiotics in the treatment of listeriosis.

Materials and methods

Bacterial strains, isolation and molecular characterisation

A total of 52 *L. monocytogenes* isolates from 12 MLST subtypes (ST) commonly associated with the food chain were chosen (up to 5 isolates per ST) from a variety of sources (dairy, meat, vegetable, mixed food and environment; Supplementary Table 1; appendix B). Isolates were stored in a -80°C freezer, and resuscitated on Brain Heart Infusion (BHI, Oxoid, Australia) agar at 37 °C for 24 h, prior to experimental manipulations.

Phenotypic characterisation

Antimicrobial sensitivity

The sensitivity of *L. monocytogenes* strains to five antibiotics used for the treatment of listeriosis was determined on Muller Hinton Agar (Oxoid, Australia) supplemented with 5 % (v/v) defibrinated sheep blood (MHSBA; Thermo Fisher Scientific, Australia). Ciprofloxacin, trimethoprim/sulfamethoxazole and penicillin G (0.002-32 µg/mL) along with gentamicin and amoxicillin (0.016-256 µg/mL), were assessed. Bacterial suspensions adjusted to 0.5 McFarland in maximum recovery diluent (MRD) were inoculated onto MHSBA using a sterile swab in three directions and incubated at 37 °C for 24-48 h. The Minimum Inhibitory Concentration (MIC) for each antibiotic was assessed using Etest strips (Biomerieux, Australia) and MIC values were interpreted according to breakpoints provided by the Clinical Laboratory Standards Institute (CLSI) (23, 24) or European Committee on Antimicrobial Susceptibility Testing (EUCAST) (25) or the literature (26) for *L. monocytogenes* where available; or staphylococcal species MICs were used in the absence of breakpoints from the aforementioned sources. The MIC value for susceptibility was defined as ≤ 1 µg/mL for amoxicillin, gentamicin and ciprofloxacin, ≤ 2 µg/mL for penicillin and ≤ 0.064 µg/mL for

trimethoprim/sulfamethoxazole. The control strains used were *S. aureus* ATCC 29213 and *Streptococcus pneumoniae* ATCC 49619.

Disinfectant and heavy metal sensitivity

Benzalkonium chloride (BC) (Sigma Aldrich, Australia) was used to determine sensitivity of *L. monocytogenes* strains to an important industrial quaternary ammonium compound disinfectant, using a broth microdilution method with the following modifications: briefly, *L. monocytogenes* strains were grown overnight in Mueller Hinton broth (MHB) and diluted to ~10^3 CFU/mL, then 190 µL was inoculated into 96 well micro-titre plate. For each BC stock concentration, 10 µL was added to the micro-titre plates to achieve final concentrations of 50, 40, 30, 20, 10, 5, 2.5, 2, 1.5, 1 and 0.5 µg/mL. All plates were incubated at 37 °C for 24 h. Growth was monitored immediately following inoculation (T0) and again at 24 hr (T24) at OD_{600} using EnSpire™ multilabel plate reader 2300 (PerkinElmer, Singapore). The T24 reading was subtracted from T0 to determine if isolates were capable of growth. The bacteriostatic or bactericidal effect of BC was tested for any isolate with growth under OD_{600} 0.1 with 10 µL spotted onto Brain Heart Infusion agar (BHIA) and incubated at 37°C. After 24 h plates were checked for growth or no growth. A minimum of two biological replicates were performed.

Cadmium chloride ($CdCl_2$) was used to determine *L. monocytogenes* sensitivity to the heavy metal cadmium, using a previously optimized method (27). Mueller Hinton agar (Oxoid, Australia) was supplemented with $CdCl_2$ (Sigma Aldrich, Australia) at the following final concentrations: 10, 35, 40, 70, 140 and 150 µg/mL. Isolates were grown overnight on BHIA and a 1 µL loopful was inoculated into 2 mL phosphate-buffered saline (PBS) and vortexed until completely suspended. The PBS and isolate solution were streaked with a cotton swab onto the $CdCl_2$ plates in three directions and incubated at 37°C. Plates were visually assessed for growth or no growth after 48 h. Two biological replicates were performed.

Genomic characterisation

Genomic characterisation was predominately performed in Geneious (2020). Genes of interest were downloaded from NCBI with searches performed in Geneious using Megablast or tblastn, with positive results for hits displaying >85 % query coverage and pairwise identity. A phylogenetic tree was created based upon raw reads using Snippy and Snippy-core (28) in Galaxy Australia (29) utilising the genbank file of isolate 7943 as the reference genome and reconstructed with RAxML (v8.2.4) (30), utilising substitution model 'GTRCAT' and the remainder with default parameters.

Genetic determinants of virulence potential and FPE stress survival

The LIPI-1 virulence cluster and a selection of genetic determinants identified in the literature were chosen to assess the potential of the isolates to survive various stress conditions encountered within the FPE, and the potential to cause disease should a contaminated food product be consumed. The protein or gene was downloaded from NCBI and a BLAST search of the genetic determinants occurred in Geneious utilising the above criteria. EasyFig 2.2.5 (31) was used to visualise gene comparisons.

Antimicrobial and virulence gene databases

Mass screenings of acquired antimicrobial and virulence genes were performed using Abricate in Galaxy (v1.0.1) (32) against the associated databases NCBI AMRFinder Plus (33), CARD (34, 35), ARG-ANNOT (36), Resfinder (37) and VFDB (38).

Mobile Genetic Elements characterisation

Plasmids were identified using PlasmidFinder 2.1 against the Gram-positive database (39). Prophage elements were identified using the online platform PHASTER (40, 41). Draft nucleotide sequences were utilised for both analyses. Confirmation of plasmid and prophage results were performed in Geneious (2020), through contig interrogation and read-mapping.

Comparison of closed plasmids was visualised using BRIG (42), with the following combinations: ST8 plasmids with pLM1686 as the reference plus an additional section from p7922 from this study, ST121 and ST321 utilising pLM6179 for reference and ST3, ST9, ST155 and ST204 were compared to pN1-011A and pR479a plasmids.

Results

Genomic composition of L. monocytogenes isolates

An overview of the genomic composition of the 52 *L. monocytogenes* isolates included in this study is shown in Supplementary Table 2; appendix B. The draft genome sizes ranged between 2.61 and 3.08 Mb, with the GC percentage between 37.7 and 38.1 %. The number of coding DNA sequences ranged from 2,668 to 3,165.

Cadmium Chloride and Benzalkonium Chloride Phenotypes

The sensitivity of the 52 *L. monocytogenes* strains to various concentrations of BC and $CdCl_2$ is shown in Figure 1. When assessed against BC only two isolates (7544 and 7546) were unable to grow at the lowest concentration (0.5 µg/mL), however when subsequently spotted onto BHI agar they were able to produce colonies indicating BC at 0.5 µg/mL had a bacteriostatic effect on these two isolates. A minimum inhibitory concentration of 1 µg/mL was observed for 22 isolates, 1.5 µg/mL and 2.5 µg/mL for one isolate each, 5 µg/mL for 24 isolates and 10 µg/mL for two isolates. No isolates were able to grow at 20 µg/mL or above.

Assessment against varying concentrations of $CdCl_2$ resulted in 10 isolates unable to grow at the lowest $CdCl_2$ concentration (10 µg/mL). Ten isolates were able to grow at 10 µg/mL, 6 isolates grew at 40 µg/mL, 21 isolates grew at 70 µg/mL and 5 isolates were able to grow at 140 µg/mL. Isolate 7920 contained a $CdCl_2$ resistant gene, however it was only able to grow to 10 µg/mL. No isolates were able to grow at the highest concentration (150 µg/mL). There were also nine isolates which had no *cadA* genes but were able to grow at 10 ug/ml.

Antimicrobial susceptibility

Five antibiotics used for the treatment of listeriosis were tested against the *L. monocytogenes* isolates (Figure 1). All the *L. monocytogenes* isolates displayed sensitivity to the antibiotics tested in this study (amoxicillin, gentamicin, penicillin, ciprofloxacin and trimethoprim/sulfamethoxazole).

Food production stress determinants

All isolates were assessed for the presence of genetic determinants relating to various stress conditions experienced within the food production environment, used as a mitigation strategy to reduce growth and/or survival of *L. monocytogenes*. These stress conditions included thermal treatment, low temperatures, acidification, oxidation, osmotic stress, the use of bacteriocins or nisin, and high hydrostatic pressure (HHP). A screening database of genetic determinants associated with each condition identified within the literature was selected to determine the potential of strains to survive within the FPE. The genetic determinants selected for heat, acid, cold, osmotic, bacteriocin/nisin and HHP stresses were present in all isolates (Supplementary Table 3; appendix B). The stress survival islets (SSI) were also assessed, with SSI-1 present in 34 of the 52 isolates (65.4 %) from ST3, 7, 8, 9, 12, 155, 204 and 321 (Figure 1). The five isolates from ST121 were positive for SSI-2 (9.6 %), and an SSI genotype harbouring an *LMOf2365_0481* gene homolog was present in 13 of the 52 isolates (25.0 %) from ST1, 2 and 101.

Cadmium and disinfectant resistance genes

The *L. monocytogenes* isolates were analysed for the presence of cadmium resistance genes *cadA1C-A6C* (Figure 1). There were 19 isolates which had no *cadA* genes present; the *cadA1* gene was present in 20 isolates, *cadA2* was represented in five isolates, four isolates had *cadA4* and five isolates had *cadA5* present. No isolates had the *cadA3* or *cadA6* gene. Isolate

7929 had two *cadA* genes present, *cadA2* and *cadA4*. Two *cadA* genes were also present in isolate 7533, *cadA2* and interestingly, it also contained a transposon with a novel *L. monocytogenes* cadmium resistance gene, referred to here as *cadA7*. A nucleotide BLAST search of NCBI nucleotide database identified four other *L. monocytogenes* strains also contain this transposon and the novel *cadA7* gene homologue. This transposon was also identified in Enterococcaceae strains suggesting direct or indirect horizontal gene transfer occurring between *Enterococcus* and *Listeria* (Figure 2). No other isolates within this study contained the *cadA7* gene. Amino acid sequence identity of the published cadmium genes and the novel *cadA7* gene was determined, with *cadA7* sharing the highest amino acid percentage identity with *cadA2*, 75.74 % (Supplementary Figure 1; appendix B); however, when compared to *E. faecalis* ATCC 29212 genome the novel *cadA7* displayed 100 % coverage and 99.95 % nucleotide identity.

A variety of genes and mechanisms providing resistance to disinfectants were assessed against the isolates in this study (Figure 1). All isolates were found to contain the *mdrL* and *lde* efflux pumps. The *bcrABC* cassette was present in 13 isolates covering ST1, 3, 9, 155, 204 and 321. Six isolates in total were positive for *ermB* and *qacH* from ST8, 9 and 121. The *emrC* gene was present in six isolates, one isolate from ST7 and all the isolates from ST101. The ST101 isolates were the only whole ST group in which all contained the same disinfectant resistance gene.

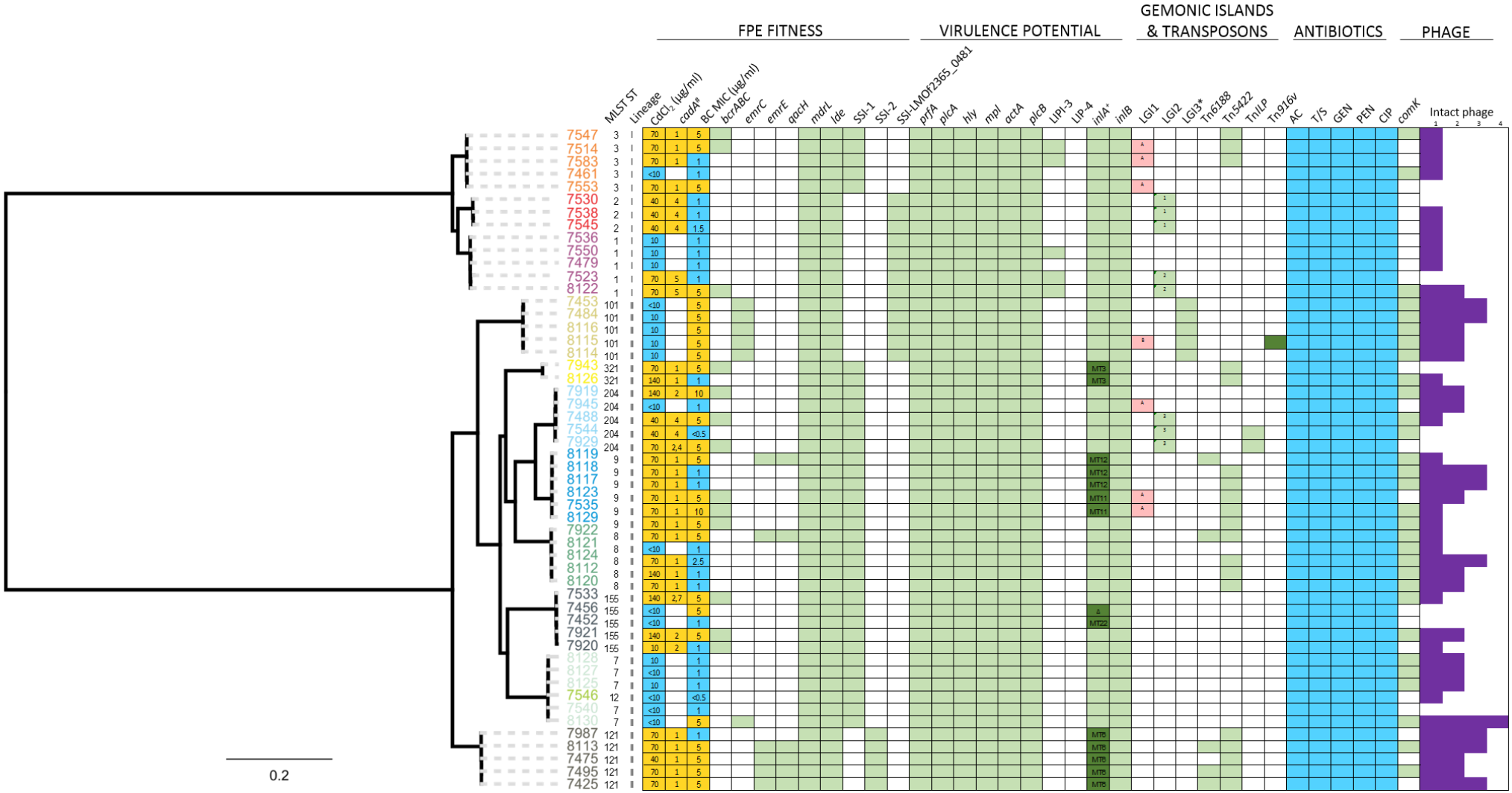
FPE FITNESS
VIRULENCE POTENTIAL
GEMONIC ISLANDS & TRANSPOSONS
ANTIBIOTICS
PHAGE
MLST ST
Lineage
CdCl2 (µg/ml)
cadA*
BC MIC (µg/ml)
bcrABC
emrC
emrE
qacH
mdrL
lde
SSI-1
SSI-2
SSI-LMOf2365_0481
prfA
plcA
hly
mpl
actA
plcB
LIPI-3
LIP-4
inlA*
inlB
LGI1
LGI2
LGI3*
Tn6188
Tn5422
TnlLP
Tn916v
AC
T/S
GEN
PEN
CIP
comK
Intact phage
0.2

Figure 1. Phylogeny and genetic determinants of 52 *L. monocytogenes* isolates relating to survival within the FPE, virulent potential and therapeutic treatment potential. Character designations are as follows: #, numbers designated *cadA* gene type; ^, pLi0048 – elements of the pLi100 are present however we were unable to close or identify full plasmid. This plasmid is known to carry cadmium resistant genes; +, *inlA* PMSC type; ∆, 70 AA deletion; A, phage insert in the *lmo1703-lmo1702* region; B, transposon insert in the *lmo1703-lmo1702* region (Tn*916* variant); 1, LGI2 insert within the *EGD-e LMO2257* gene; 2, LGI2 variant; 3, LGI2 insert in the *yfbR* gene; *, LGI3 lacking the *cadA1C* cassette; yellow, resistant phenotype; light green, gene is present; dark green, gene is present – does not match wildtype; light red, LGI1 is absent however there is alternative genes present within the *lmo1703-lmo1702* region; blue, sensitive phenotype; purple, number of intact phage regions present.

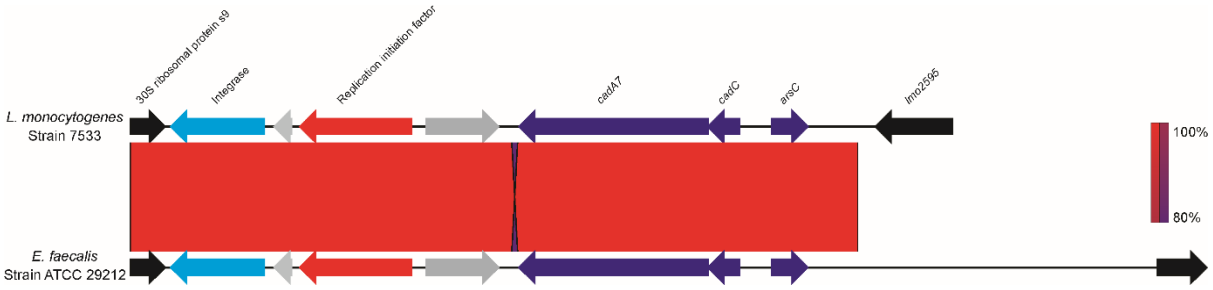

Figure 2. The novel *cadA7* gene. Transposon identified in isolate 7533 inserted between 30S ribosomal protein S9 and *lmo2595*, compared to *E. faecalis* ATCC 29212. Integrase genes are in light blue, replication genes are in red, heavy metal resistance genes are in purple. Hypothetical genes or those with an unknown function are shaded grey. Nucleotide sequence identity of transposon where shared, ranged from 80 to 100 % as depicted by the percentage homology bar.

Plasmids, Prophages and Transposons

Plasmid replicons were identified in 26 of the 52 isolates with PlasmidFinder. The identified regions were further interrogated in the draft genomes. A total of 13 closed plasmids and 12 draft open plasmids were identified (Table 1). Plasmids were present in ST3, ST8, ST9, ST121, ST155, ST204 and ST321 isolates (Figure 3). ST121 was the only group in which

plasmids were found in all five isolates and displayed a 95.9 % pairwise identity with pLM6179, however only three of these plasmids were closed following sequence analysis.

Table 1. Plasmids identified among isolates in this study.

		Plasmid		
Isolate	MLST	Closed/ Open	Size (bp)	GC (%)
7514	3	Open	59,826	35.2
7547	3	Open	32,307	36.1
7553	3	Closed	4,176	34.1
7583	3	Open	56,146	36
7922	8	Closed	88,290	36.5
8112	8	Closed	79,144	36.7
8120	8	Open	85,394	36.9
8124	8	Closed	79,180	36.7
8117	9	Closed	25,550	36.5
8118	9	Closed	25,550	36.5
8119	9	Closed	25,550	36.5
8123	9	Open	49,141	36.3
8129	9	Open	49,281	35.8
7425	121	Closed	62,207	36.5
7475	121	Open	60,666	36.7
7495	121	Open	62,191	36.5
7987	121	Closed	60,923	36.6
8113	121	Closed	62,207	36.5
7533	155	Open	64,751	38.1
7920	155	Closed	77,756	37.5
7921	155	Closed	80,184	37.4
7488	204	Open	48,687	37.4
7919	204	Closed	38,191	37.3
7929	204	Open	91,345	37.7
7943	321	Open	66,904	36.5
8126	321	Open	60,124	36.7

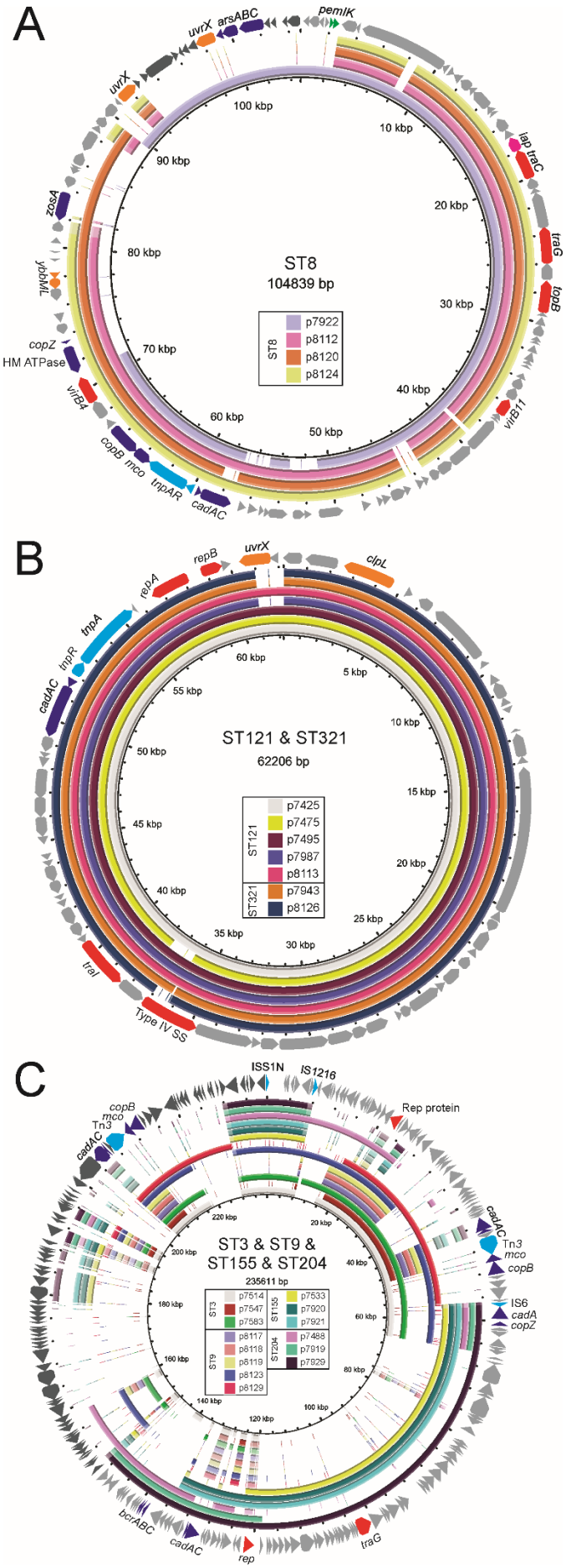
A
ST8
104839 bp
p7922
p8112
p8120
p8124
pemIK
uvrX
arsABC
uvrX
zosA
yobMI
copZ
HM ATPase
virB4
copB
mco
tnpAR
cadAC
virB11
traG
lobB
lap
traC
B
ST121 & ST321
62206 bp
p7425
p7475
p7495
p7987
p8113
p7943
p8126
repB
uvrX
clpL
repA
tnpA
tnpR
cadAC
traI
Type IV SS
C
ST3 & ST9 &
ST155 & ST204
235611 bp
p7514
p7547
p7583
p7533
p7920
p7921
p8117
p8118
p8119
p8123
p8129
p7488
p7919
p7929
ISS1N
IS1216
Rep protein
copB
mco
Tn3
cadAC
Tn3
mco
copB
IS6
cadA
copZ
bcrABC
cadAC
rep
traG

Figure 3. BRIG comparison of plasmids identified in 26 isolates within this study. Each ring represents the plasmids of the individual isolates. The plasmid pangenome reference contains all the unique genetic features of the closest related plasmid identified in NCBI and segments of plasmids from the respective pST groups from this study which are not represented in NCBI, combined into a single contig reference (outside ring). **(A)** pST8 utilises the genome of pLM1686 (MK134858) (light grey) and p7922 (dark grey) as reference pangenome. **(B)** pST121 and pST321 utilises the p6179 as reference genome. **(C)** pST3, pST9, pST155 and pST204 utilises the genome of pN1-011A (light grey) and pR479a (dark grey) as reference pangenomes. Annotated genes are colour coded to represent genetic markers as follows: red – replication, light Blue – transposases, dark Blue – heavy metals, orange – stress response, pink – invasion associated, green – toxin/antitoxin and grey – hypothetical proteins.

Genes shared across the plasmids, and not restricted to a single ST and included heavy metal and disinfectant resistance genes including the *bcrABC* operon, the *cadAC* operon, and genes for copper, zinc and arsenic resistance; stress response genes including UV damage repair protein, oxidative and heat stress response genes; invasion related genes; toxin/anti-toxin genes; genes involved in DNA replication, translation, recombination and conjugation; transposon genes; however most genes were hypothetical proteins.

Phaster identified 52 intact phage regions across 43 isolates. Nine isolates had no phage regions, most isolates had a single phage region, two regions were identified in 15 isolates and three and four regions identified in four and one isolate, respectively. An additional analysis of the *comK* phage insertion site identified 21 isolates with a full length *comK* gene and 31 isolates with a *comK* prophage disruption (Figure 1). Transposon elements were identified in 27 of the 52 isolates. Transposon Tn*6188* was present in five isolates, Tn*5422* was identified in 20 isolates, Tn*ILP* was present in two isolates and Tn*yfbR* was identified in six strains. Seven isolates contained two transposons.

Virulence determinants and genomic islands

The *Listeria* pathogenicity and genomic islands were assessed against the panel of isolates (Figure 1). The LIPI-1 virulence cluster was present in all isolates, along with the *inlB* gene. The LIPI-3 element was found in three isolates (7523, 7550 and 8122) from ST1 and two isolates (7514 and 7583) from ST3. No isolates harboured LIPI-4; this island has only been identified in CC4 isolates, which were not included in this study. The LGI2 was present in eight isolates, in either one of two insertion locations; within the *LMOSA2140* (homolog of *LMOf2365_2257*) gene originally identified in the strain ScottA (43), or within the *yfbR* gene (44). The three isolates with the *LMOSA2140* LGI2 insertion region were from ST2 and three ST204 strains contained the *yfbR* LGI2 insert. Interestingly, an LGI2 variant was also identified from two ST1 isolates within a transmembrane protein that displays distant homology to *ydbT* gene. This LGI2 variant harbours and additional gene, a *metC* homolog, within the LGI2 region (Figure 4). The recently reported *Listeria* genomic island 3 was partially identified in the five ST101 isolates only; however, they were missing the 6,248 bp region containing the *cadAC* homolog, recombinase and Tn*3* family transposase.

Internalin A (inlA) Analysis

The *inlA* gene was assessed for the presence of mutations resulting in premature stop codons (PMSCs), truncation or a full length *inlA* gene (Figure 1 and Supplementary Figure 2; appendix B). Thirty-eight of the isolates contained a full length *inlA* gene. Thirteen isolates contained PMSCs. All ST121 isolates (7425, 7475, 7495, 7987 and 8113) contained mutation type 6 at AA 492, as previously described (45). Isolates 8123 and 7535 contained a PMSC at AA 685, resulting in mutation type 11. Both these isolates are ST9, from which this mutation type has been previously associated with (45). Mutation type 12, the result of a PMSC at AA 576 was identified in three isolates from ST9 (8117, 8118 and 8119) and mutation type 3, the result of a PMSC at AA 700, was identified in isolates 8126 and 7943 from ST321. A novel PMSC was identified in isolate 7452, the result of a frame shift mutation from an AG insertion at nucleotide

position 183 producing an *inlA* gene of 67 AA in length. Isolate 7456 contained an in-frame 70 AA deletion within the B-repeat region (B- repeat region 2 and 3).

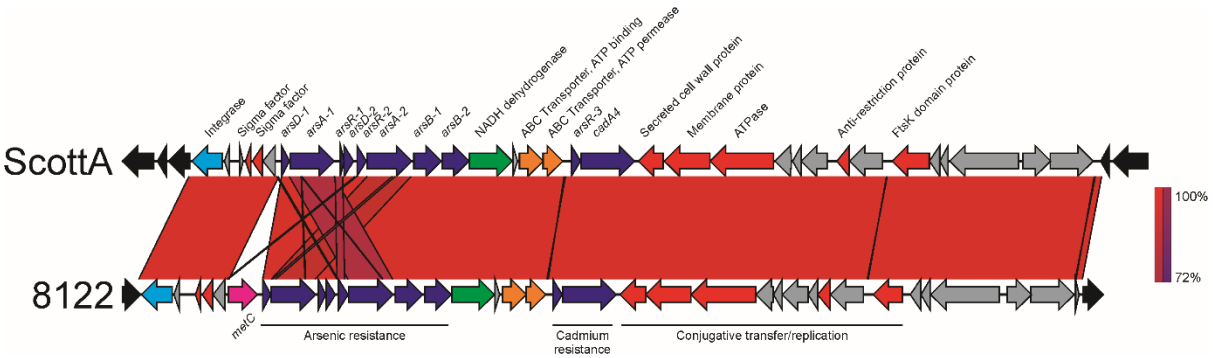

Figure 4. LGI2 variant identified in isolate 8122. A variant of LGI2 inserted in a transmembrane protein within isolate 8122 and 7523. Annotated genes are colour coded to represent genetic markers as follows: black – flanking genes, light blue – integrase, dark blue – heavy metal and antimicrobial resistance, orange – metabolism and transport, red - transposon system and regulatory genes, pink - virulence , green – stress resistance , grey – hypothetical proteins. Sequence identity where shared, ranged from 72 to 100 % as determined by the percentage homology bar.

Listeria genomic island 1 analysis

None of the isolates harboured the *Listeria* genomic island 1 (Figure 1). To confirm this, we manually inspected the hypervariable region between *lmo1703-lmo1702* locus, an RNA methyltransferase gene and the fosfomycin resistance gene *fosX* respectively, for inserts in all isolates. A phage (ϕRNA-MT) insert was present in six of the isolates (7514, 7535, 7553, 7583, 7945 and 8123) and a novel insert was harboured in isolate 8115. This novel insert was not present in any of the other isolates. A BLAST search resulted in a 64 % query coverage and 90.73 % nucleotide identity with Tn*916* from *Bacillus subtilis* (which has over 98 % identity with Tn*6198* of *L. monocytogenes* TTH-2007) and a 64 % query coverage and 91 % identity with an integrated chromosomal element ICESpnIC1 identified in *St. pneumoniae* isolate 9611+04103 (Figure 5). This Tn*916* variant insert between *lmo1703* and *lmo1702* in isolate

8115 is approximately 23,275 bp in length, has a GC content of 37.5 % and contains an clp protease ATP-binding subunit *clpA*, *ftsK/SpoIIIE* family protein, Tn*916* transcriptional regulator, anti-restriction protein, lipoprotein, *XRE* family transcriptional regulator, efflux *ABC* transporter, site-specific recombinase with the remaining genes being hypothetical proteins.

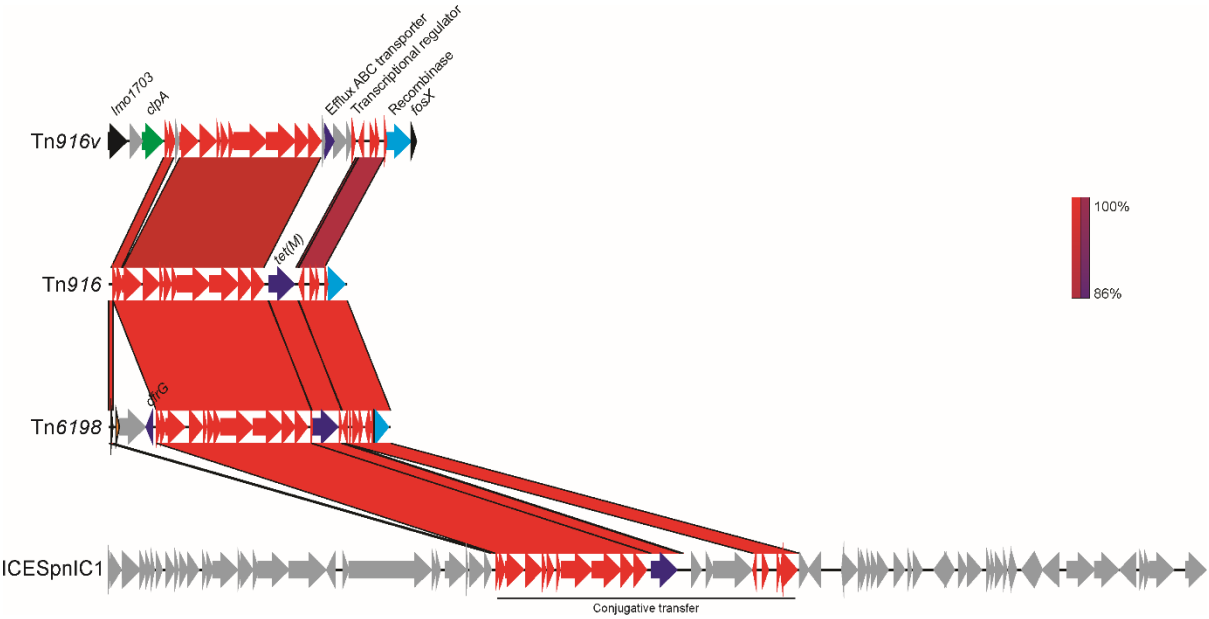

Figure 5. Novel insert identified in isolate 8115. The insert was identified in the hypervariable region of isolate 8115 between genes *lmo1703-lmo1702*, compared to Tn*916*, Tn*6198* and *S. pneumoniae* 9611+04103 ICESpnIC1. Sequence identity where shared, ranged from 78 to 100 % as determined by the percentage homology bar. EGD-e flanking genes, *lmo1703* and *lmo1702* are shown in black; red genes are transposon systems and regulatory genes, light blue – integrase, dark blue – heavy metal/antimicrobial resistance, green – stress response and grey – hypothetical proteins.

Antimicrobial resistance determinants

The *L. monocytogenes* isolates were analysed for the presence or absence of a variety of antimicrobial resistance genes associated with conferring resistance to frequently used antibiotics in the treatment of listeriosis or other diseases (Supplementary Table 4; appendix B). Resistance genes from the following selected antibiotics classes: trimethoprim, tetracycline

except for the *tetA-like* resistance protein, vancomycin, erythomycin, amoxicillin and aminoglyosides along with the pencillin *mecC* protein were not detected in any isolates. The AMR genes selected relating to fluroquinolones (second generation), penicillin (except *mecC*), sulfonamide, fosfomycin, lincomycin, fusidine, quinolone and cephalosporins were present within all isolates. There were no AMR genes or class specific to an isolate, lineage or ST. Potential resistance mechanisms beyond known AMR genes were not examined.

Discussion

This study utilised phenotypic and genotypic analyses of whole genome sequences to assess the potential of *L. monocytogenes* to survive within the FPE, cause disease and provide insights into antimicrobial resistance relevant to control in the FPE, or the treatment of human infection.

Virulence potential

Clonal complex 1 (CC1) and 2 (CC2) are well established as being associated with clinical infections (46, 47), and therefore the presence of genomic regions relating to hypervirulence in food isolates is of interest. In this study ST1 and ST2 isolates did not contain the SSI-1 or SSI-2 islands, however, the SSI-alternative, *LMOf2365_0481* homologue, was present in all isolates. A study by Harter and colleagues (9) reported the presence of this SSI-alternative to be common among clinical strains, however its function at this stage is undetermined. While CC1 and CC2 did not contain SSI-1 or SSI-2, the isolates did contain all but one of the stress resistance-associated genes screened, with a role in heat, cold, acid, osmotic, oxidative or nisin stress response, suggesting that they are capable of surviving within the FPE, or in food. Horlbog et al (48) found CC1 strains were able to recover more quickly after salt stress, suggesting these strains could be able to proliferate faster within food. All isolates of CC1 and CC2 contained full length *inlA* genes; three of the CC1 isolates contained LIPI-3, suggestive of increased virulence potential.

Within Australia, CC3 and CC204 are widely distributed (49), with these strains analysed in this study all containing LIPI-1, full length *inlA*, SSI-1, and all the various processing related stress genes. Neither CC3 nor CC204 contained the LGI1, however, three of the CC3 strains and one of the CC204 strains contained a phage insert in the LGI1 hypervariable position between *lmo1703* and *lmo1702*. Interestingly, two of the CC3 isolates contained the phage insert instead of LGI1. Increased bacterial colonisation and hypervirulence has been reported in isolates which encode a full length *inlA*, LIPI-1 and LIPI-3 (46, 47), suggesting the isolates in this study which contain these have increased virulence potential, and with the addition of SSI-1 may also have increased FPE fitness. The CC204 strains which were negative for LGI1 and the ϕRNA-MT phage insert, contained LGI2. *Listeria* genomic island 2 contains cadmium and arsenic resistance genes, providing increased environmental survival potential; interestingly, also in both this study and Lee et al (50), LGI2 was common in CC2 strains, which are also prevalent in human cases. Although LGI2 requires further characterisation, it is prevalent in clonal complexes linked to hypervirulence and has been suggested to contribute to virulence (50).

Premature stop codons and mutations in the *inlA* gene resulting in secretion of *inlA* instead of being attached to the bacterial cell wall have been associated with reduced invasion and virulence ability (51-53). In this study, 13 isolates were identified to carry one of five types of mutations, suggesting these isolates may have a reduced virulence potential. We identified a novel PMSC at 67 AA resulting from an AG insertion at position 183 bp producing a frameshift, referred to as mutation type 22. This mutation occurs within the signal cap region of the *inlA* protein. Mutation types 4 and 15 occurring at AA positions 9 and 77 respectively have been shown to affect invasion ability (45), indicating mutation type 22 has the potential to also have reduced invasiveness, however, virulence assays will be required to confirm this theory. In addition, a 70 AA deletion within the B-repeat region was identified in isolate 7456. Deletion of the B-repeat region between AA 517 and 707 by Lecuit et al (54) resulted in similar

invasiveness level to those with the WT *EGD-e inlA* protein, suggesting the 70 AA deletion within the B-repeat region does not contribute to a strains ability to invade cells. Virulence assays will be required to determine the invasiveness of mutation type 22 and the 70 AA deletion. All ST121 isolates contained the type 6 mutation and ST321 isolates contained mutation type 3. In addition, all but two *inlA* mutant isolates had cadmium resistance, contained either SSI-1 or SSI-2, and harboured plasmids. All of these isolates harboured LIPI-1. The majority of *inlA* mutants are commonly associated with the FPE and food isolates (51, 55), therefore the presence of these genes in the mutant *inlA* isolates are suggestive of increased survival within the FPE.

FPE survival potential

Agricultural practices and industrial pollution have resulted in increased levels of various heavy metals in the environment, and as such bacteria require resistance determinants in order to tolerate these substances, particularly heavy metals which are not required for cellular processes. Cadmium resistance determinants are widely distributed and are commonly associated with *L. monocytogenes* strains repeatedly isolated from food sources (56). In this study, 32 isolates were capable of growing at levels above 40 µg/ml $CdCl_2$, which was associated with the presence of at least one cadmium resistance determinant, with similar results observed in previous studies (27, 43, 57-59). Interestingly, in this study we did not identify the *cadA3* resistance determinant which is present as an integrating chromosomal element in a variable genomic region, that in other strains may contain diverse cassettes like LIPI-3 (60).

The novel *cadA7*, identified in this study, contained all three key motifs, DKTGT, CPC and CTNCA, characteristic of the *cadA* protein family (56, 61). Parsons et al (56) identified an amino substitution in the CTNCA → CANCA motif in *cadA4* suggesting this substitution most likely, in conjunction with other elements, influences *cadA4*'s reduced tolerance of 35 µg/mL to cadmium. In this study, the three key motifs of the novel *cadA7* matched the *cadA1-cadA3*

sequences, which are purportedly associated with cadmium resistance of 140 µg/mL or higher, potentially suggesting *cadA7* may confer similar levels of resistance (56). The single isolate harbouring *cadA7* in this study also contained *cadA2*; as such, further research is required to establish the resistance level conferred by *cadA7*, and to determine if it has a potential role in virulence.

The presence of disinfectant resistance genes in this study was associated with resistance to BC (≥ 5 µg/mL) in all but two isolates, with 50 % of the isolates displaying a MIC of 5 µg/mL or higher. The capability of isolates to grow at higher levels of disinfectants like BC is being increasingly reported (62, 63). In addition, the *L. monocytogenes* strains' tolerance to disinfectants has been correlated with cadmium resistance and increased survival within the FPE being associated with subinhibitory levels of disinfectants (63-65). Therefore, an evaluation of the level of resistance to cadmium and BC is important to understand the survival potential *L. monocytogenes* may have in the FPE.

Genomic islands have the potential to contain genes to improve the fitness of an isolate, while also being implicated in potential horizontal gene transfer (66); therefore, the presence of these islands might lead to increased FPE survival or pathogenic potential. Of the genomic islands identified in *L. monocytogenes*, LGI1 and LGI3 have been associated with survival and persistence in the FPE (66, 67), with LGI2 potentially providing increase survival and persistence within the FPE as well as virulence potential (43, 50). In this study, LGI2 was the only full-length island present in six isolates, inserted within one of two genes, *LMOSA2140* or *yfbR*. This has the potential to provide increased virulence and environmental fitness. In addition, an LGI2 variant was identified in two ST1 isolates within a transmembrane protein that displays distant homology to the *ydbT* gene from *Bacillus*. The *ydbT* in *Bacillus subtilis* strains has been reported to provide resistance to bacteriocins produced by *B. amyloliquefaciens*, an important function particularly in natural environmental reservoirs like soil (68). The LGI2 variant shows high homology with LGI2 and maintains the arsenic and

cadmium resistance determinants along with various metabolism, transport, stress resistance, transposon and regulatory genes. However, the LGI2 variant contains an additional Cystathionine β-Lyase (*metC*) gene. A previous study demonstrated that, when disrupted in *Salmonella*, *metC* was shown to reduce strain virulence in a mouse model (69). This suggests the LGI2 variant may play a role in virulence as well as survival within food and the food environment; however, this needs to be further confirmed experimentally.

In this study, the LGI3 variant which lacks the *cadA1C* cassette was identified in all ST (CC) 101 isolates. The LGI3 element was first identified in CC101 isolates by Palma et al (66) and found to harbour a *cadA1C* cassette; however, a search of the NCBI genome database identified a smaller LGI3 variant lacking the *cadA1C* cassette in the *L. monocytogenes* strain ATCC 51775 (ST222). In comparison, the CC101 isolates from this study also contained the LGI3 variant, suggesting CC101 strains may display either of the LGI3 genotypes.

Instead of LGI1, six isolates contained a phage insert and one isolate contained a Tn*916* variant insert, in the associated insertion locus. This Tn*916* variant shares similarity with Tn*916* and Tn*6198*; however, it lacks the tetracycline (*tetM*) and the trimethoprim (*dfrG*) resistance genes. Interestingly, an efflux ABC transporter is present on the Tn*916* variant, which shares homology to efflux systems. Further experimental work is required to determine its function in this transposon, and the ability of this transposon to transfer to other *Listeria* strains, or other bacterial species.

Therapeutic treatment potential

Traditionally, listeriosis is treated with a β-lactam (penicillin, ampicillin or amoxicillin) either alone or in combination with an aminoglycoside, typically gentamicin (22, 70-72). For patients which have a β-lactam sensitivity, trimethoprim in combination with sulfamethoxazole is used (21, 73). This current form of chemotherapy has been deployed for decades due to slow levels of resistance acquisition observed with *L. monocytogenes*; however, increasing prevalence of

resistance is slowly developing to various antibiotics. While in this study all the isolates were sensitive to the five clinically relevant antibiotics tested, there has been reports in the literature of resistance to gentamicin, penicillin, ciprofloxacin and trimethoprim/sulfamethoxazole along with a variety of other antibiotics and importantly reports of resistance to multiple antibiotic classes (74-78). Our study provides a timely contribution to the current state of AMR in *L. monocytogenes* and does not highlight any resistance concern among food isolates in this study.

In this study, we identified a novel cadmium gene, *cadA7* as part of a transposon insert, a variant of LGI2, as well as a novel insert in the hypervariable region LGI1, the latter sharing similarity to a Tn*916* transposon. The identification of these novel genes and inserts contributes to our understanding of the *L. monocytogenes* pangenome, in particular to elements relating to survival ability and pathogenic potential. The isolates analysed in this study showed potential to survive and persist within the FPE, with all isolates containing one of the SSIs, various genes relating to stressors present in the FPE to reduce bacteria, in addition to a high portion of strains containing cadmium or disinfectant resistance genes. Hypervirulent strains of *L. monocytogenes* have been previously reported, with some isolates from CC1 and CC3 in this study harbouring genes associated with this virulence status, suggesting a concern to public health.

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CHAPTER 5

Novel biocontrol methods for *Listeria monocytogenes* biofilms in food production facilities

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Abstract

High mortality and hospitalisation rates have seen *Listeria monocytogenes* as a foodborne pathogen of public health importance for many years and of particular concern for high risk population groups. Food manufactures face an ongoing challenge in preventing the entry of *L. monocytogenes* into food production environments (FPEs) due to its ubiquitous nature. In addition to this, the capacity of *L. monocytogenes* strains to colonise FPEs can lead to repeated identification of *L. monocytogenes* in FPE surveillance. The contamination of food products requiring product recall presents large economic burden to industry and is further exacerbated by damage to the brand. Poor equipment design, facility layout and worn or damaged equipment can result in *Listeria* hotspots and biofilms where traditional cleaning and disinfecting procedures may be inadequate. Novel biocontrol methods may offer FPEs effective means to help improve control of *L. monocytogenes* and decrease cross contamination of food. Bacteriophages have been used as a medical treatment for many years for their ability to infect and lyse specific bacteria. Endolysins, the hydrolytic enzymes of bacteriophages responsible for breaking the cell wall of Gram-positive bacteria, are being explored as a biocontrol method for food preservation and in nanotechnology and medical applications. Antibacterial proteins known as bacteriocins have been used as alternatives to antibiotics for biopreservation and food product shelf life extension. Essential oils are natural antimicrobials formed by plants and have been used as food additives and preservatives for

many years and more recently as a method to prevent food spoilage by microorganisms. Competitive exclusion occurs naturally among bacteria in the environment. However, intentionally selecting and applying bacteria to effect competitive exclusion of food borne pathogens has potential as a biocontrol application. This review discusses these novel biocontrol methods, their use in food safety and prevention of spoilage, and examines their potential to control *L. monocytogenes* within biofilms in food production facilities.

Introduction

Listeria monocytogenes is a Gram-positive, rod shaped, facultative anaerobe capable of causing food borne illnesses particularly in high risk population groups including the elderly, immune compromised, pregnant women and neonates (1). While *L. monocytogenes* associated illness is not as common as that of other food borne pathogens like *Salmonella, Campylobacter* or *Escherichia coli*, its mortality rate can be considered the highest. Approximately 30 % of invasive listeriosis cases lead to mortalities with most requiring hospitalisation therefore demanding *L. monocytogenes* be considered as a food borne pathogen of public health importance (2, 3). Due to its ubiquitous nature, *L. monocytogenes* poses a food safety risk as it is frequently introduced into the processing environment through raw ingredients. *L. monocytogenes* can adhere to a variety of abiotic surfaces with some strains persisting for numerous years and acting as a source of continuous cross contamination (4-6).

Due to significant food safety risks the control of *L. monocytogenes* has become a regulatory requirement that food business operators must adhere to. Regular cleaning, disinfecting and sanitising of food contact and non-food contact surfaces is required as part of a sanitation plan that also incorporates maintenance of equipment and buildings, pest control and general hygiene. In addition, the implementation of good manufacturing practices and effective hazard analysis critical control point plan aids in reducing the risk of food borne illness (7). However,

L. monocytogenes is a difficult organism to eradicate and its presence still occurs even with the best management plans (7, 8).

While the exact mechanisms can be unclear for how *L. monocytogenes* is able to persist in food production environments (FPEs) so successfully, researchers have proposed there are numerous factors at play. Poorly maintained equipment, surfaces and unhygienic factory design can result in niches containing adequate nutrients, water and protection from cleaning allowing bacteria to survive and grow while also introducing bacteria to subinhibitory levels of disinfectants (9-12). Typically disinfectants, when applied correctly, can sufficiently inhibit the colonisation of introduced planktonic cells; however, dosing failures and applying disinfectants to wet surfaces can result in equipment being inadequately disinfected and bacteria being exposed to subinhibitory chemical levels (10, 13). Incorporating desiccation processes have been shown to increase the effectiveness of disinfections procedures (14) however, an ample amount of drying time is difficult when continuous or even daily production runs are required. It is also important to note the difference between resistance, an increase in concentration or time required to exert the same reduction, and tolerance, an adaptation in a microbe's susceptibility potentially the result of exposure to subinhibitory levels (15, 16). For example, some *L. monocytogenes* strains are known to carry genes for disinfectant chemical efflux pumps, such as *qacH* and *bcrABC*. The distribution of these genes tend to vary on a strain by strain basis instead of being unique to a specific lineage or subtype (10, 17, 18). Although, it has been reported that these genes only result in tolerance to quaternary ammonium compounds at levels far below the concentrations actually used in the food industry (19). The ability to form biofilms is also a crucial factor in the survival of *L. monocytogenes.* Biofilms are comprised of numerous cells attached to each other or an abiotic surface surrounded by an extracellular matrix containing a mixture of polysaccharides, proteins and extracellular DNA (20, 21). This extracellular matrix provides a protective barrier around the internalised microbial cells from desiccation and heat, contributes to increased adhesion and is a reservoir of nutrients (22). In addition, biofilms can impede the activity of antimicrobial agents as the

matrix limits their diffusion potential, contains cells with differing susceptibility while also allowing for the acquisition of new genetic traits like those mentioned above through horizontal gene transfer. Further, biofilms typically consist of multiple species that can allow for the colonisation of transient strains or provide increased attachment and survival to strains not typically good biofilm formers (5)

The biocontrol methods movement

While tolerance to disinfectants and sanitisers is not considered as significant an issue as antibiotic resistance, their continued use and potential ineffectiveness against biofilms warrants new strategies for the control of *L. monocytogenes*. As consumers become more conscious of food safety significance, the use of novel biocontrol methods is gaining further interest. This return to biocontrol methods of microbes and plants has the potential to relieve some of the tolerance to disinfectants and decrease some of the selective pressures that their overuse has on maintaining resistance markers (5). Biocontrol methods with potential to act against listerial biofilms include bacteriophages, their endolysins, competitive bacterial species and their antimicrobial products, bacteriocins and plant derived products and will be discussed in this review.

Bacteriophages

The most abundant microorganism on earth, bacteriophages (phages) are viruses that infect bacteria for propagation, live naturally in the environment and anywhere host bacteria are found (23, 24). Phages are classified based upon their morphology (head and tail, either contractile or non-contractile, or no tail), nucleic acid (double stranded or single stranded; deoxyribonucleic or ribonucleic acid) and life cycle, which is of most relevance for biocontrol. There are two types of life cycles phages can undergo after entering the bacterial cell: the lysogenic cycle (temperate phages) or the lytic cycle (Figure 1). Phages may be capable of a lysogenic cycles that converts to the lytic cycle in unfavourable conditions, or undergo a solely

lytic life cycle. Temperate phages are not suitable as a biocontrol agent as integration into the host genome may result in increased pathogenicity through horizontal gene transfer (25, 26). In comparison, lytic phages are ideal as a biocontrol agent due to their fast-lytic action.

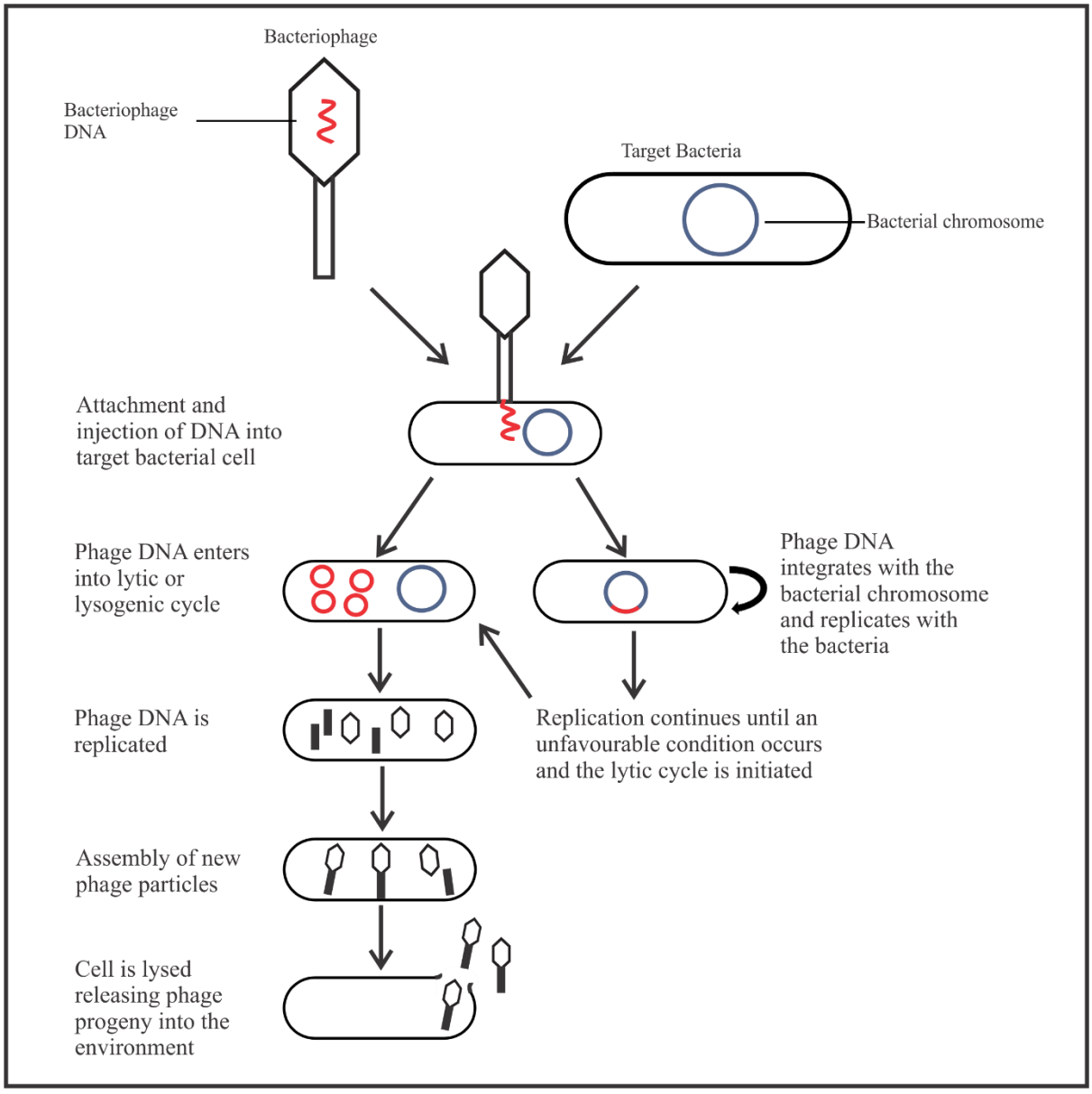

Figure 1. The two life cycles of a bacteriophage.

Although identified over a hundred years ago, interest in phages has only recently been reignited with the rise of antibiotic resistance among bacteria (25). The utility of phages has

included the treatment of diseases in humans and animals, typing of bacterial strains, decontaminating meat carcasses after slaughter, and targeted inactivation of pathogenic and spoilage bacteria on food contact and non-contact surfaces as well as surfaces of ready to eat products and during packaging and storage (25, 27). The application of phages as an innovative approach to control biofilms in the FPE is also beginning to be explored. While there has been great achievement in the use of phages from a therapeutic perspective, their success in the FPE is not as simple. Factors like the composition and structure of the biofilm, temperature, the metabolic state of the bacteria in the biofilm, the extracellular matrix in general, food components and nutrients all provide additional challenges to the effectiveness of phage application (28). While there have been some reports of phage resistance (29), it occurs more gradually than the development of antibiotic resistance as phages are able to mutate continuously, like bacteria, and resistance is further slowed by using a combination of phages active against the one bacterial species (25, 30). There is a substantial amount of research conducted on phages' ability to protect food from *Listeria*, with two commercial *Listeria* phage products, ListShield™ and Listex™ P100 approved as food preservatives with the generally recognised as safe status since 2006. However, studies investigating the efficacy of these products and other *Listeria* phages against biofilms are few, with most having focused on Listex™ P100.

Biofilm maturity has the potential to reduce the efficacy of phage treatment, as well as any control method. Various studies have examined this concept utilising preformed biofilms at various maturity levels, ranging from 24 h to two weeks, with most studies reporting a minimum 1-log reduction. Most studies to date have utilized stainless steel as the surface to form *L. monocytogenes* biofilms and examine the efficacy of bacteriophage treatments. This reflects the widespread presence of these surfaces, both food contact and non-contact in food processing environments. The success of bacteriophage treatments at inactivating *L. monocytogenes* biofilms on these surfaces, however, has shown mixed results. A number of studies demonstrated promising results when Listex™ P100 was applied to *L. monocytogenes*

biofilms on stainless steel, with reductions in the order of 5-log being achieved (31, 32). Both of these studies used an application treatment of 24 h at ambient room temperature. Iacumin et al (33) also applied Listex™ P100 for 24 h at 20 °C onto stainless steel wafers and report the complete elimination of *L. monocytogenes* biofilm. This prolonged treatment application, however, in many cases is not practical in an FPE. In addition, Iacumin et al (33) pressed the stainless steel wafer onto an agar plate to replicate the process of cross-contamination in the FPE however it did not take into consideration the phage products ability or inability to act on biofilms in the crevices or corners where these might be thicker than a flat surface.

A shorter treatment time of 2 h was applied by Sadekuzzaman et al (30) when running a similar inactivation test with ListShield™, however this was associated with a much lower inactivation of just 2-log when applied to *L. monocytogenes* biofilm on stainless steel. This was even less effective on a rubber surface, achieving a 1-log reduction in *L. monocytogenes* cell numbers. The results of Sadekuzzaman et al (30) also reflect those observed by Gutiérrez et al (34) who saw a similarly low inactivation achieved by a 4 h ListShield™ treatment, typically 1-log or less. Although the latter study did show greater inactivation with Listex™ P100 under the same treatment conditions, the Listex™ P100 commercial phage preparation showed a reduced activity range, only capable of infecting 7 of the 11 strains tested. An important aspect in phage application is the ratio of phage to bacteria known as the multiplicity of infectivity. To increase the likelihood the phage will infect the bacterium, the phage needs to be at a higher ratio than the number of target bacterial cells (32). High multiplicity of infectivity has been reported to result in efficient phage treatment with one study recommending a multiplicity of infectivity around 5 was required for adequate reductions by Listex™ P100 (32).

Apart from temperature, multiplicity of infectivity and treatment time, other factors may influence efficacy of biocontrol treatments, notably the presence of organic matter such as the food matrix. A further parameter which must be considered when examining efficacy of treatment on surfaces is the surface architecture itself, which may range from a smooth

rendered surface to a scored surface with associated crevices which may be colonised by bacteria and their biofilms. Chaitiemwong et al (35) considered both surface crevices and food matrices (diluted food residues of ham, salmon, endive or milk) when measuring the efficacy of Listex™ P100 treatment. Results suggested deeper crevice features on the surface decreased the treatment efficacy, with inactivation in the magnitude of >3-log achieved on 0.2 mm crevices compared to the max 1.4-log CFU/ml observed in crevice depth of 5 mm. Of particular note was the difference seen when comparing the food matrix, with lower inactivation observed for milk and vegetable when compared with meat or fish. Arachchi et al (36) mimicked conditions in fish processing and demonstrated the presence of fish protein led to a lower associated biofilm density compared to control stainless steel experiments when a fish protein matrix was added to the cultivation of *L. monocytogenes* biofilm on stainless steel. This highlights the complex role the food matrix may play in both biofilm formation and subsequent efficacy of bacteriophage treatment, demonstrating the need for further studies to understand the significance of food matrix on bacteriophage treatment efficacy.

Taken together, current literature detailing phage biocontrol studies directed at *L. monocytogenes,* such as those detailed above, show differing success in their ability to decrease established biofilms. The often-low reductions achieved demonstrate the challenges biofilms pose for not only bacteriophages but all control methods, but this is not to say there is no place for phages as a potential biocontrol method. As with many disinfection regimes, additional interventions such as steps to loosen biofilm or remove organic matter can increase the success of phage treatments (36). Further research considering multi-species biofilms and in-facility application will help determine the true potential of this biocontrol approach.

Endolysins

Endolysins (lysins) are hydrolytic enzymes required for bacteriophage dissemination from the host bacterial cell. They occur at the end of the lytic cycle to release the phage virions by

breaking down peptidoglycan in the bacterial cell wall in what is termed lysis from within (37, 38). Researchers have harnessed lysins through protein expression production systems, generally in *Escherichia coli*. Following purification of the lysin, it can be applied externally to the cell wall, thus not requiring phage infection, for biopreservation and biocontrol application (39). Lysins are grouped based upon the cell wall component they attack with the five main classes being N-acetylglucosaminidases, endo-β-N-acetylglucosaminidases, lytic transglycosylases, endopeptidases and N-acetylmuramoyl-L-alanine amidases (38, 39). Lysins are highly specific with a narrow spectrum of activity making them host specific with some lysins only being active on the bacterial strain the phage was isolated from (40). In addition, they are fast-acting and no development of resistance has been reported to date (38). Most research has occurred on Gram-positive bacteria using the lysis from without approach as the peptidoglycan layer is exposed. Although limited, antimicrobial activity of lysins on Gram-negative bacteria has been reported when used in conjunction with EDTA, a membrane permeabliser (37).

The antimicrobial activity of lysins has mostly focused on infection control of staphylococcal bacteria. Other applications that have been considered include use in agriculture to prevent plant disease by either intense application of cell lysates expressing a chosen lysin or development of transgenic plants by incorporation of the lysin gene into the plant genome (41, 42); as a rapid detection and imaging method of pathogenic bacteria (23, 43); and transformation of listerial bacteriophage endolysin encoding genes into dairy starter cultures as a biopreservation method (44). Antilisterial lysins isolated to date have predominately focused on the control of planktonic cells *in vitro* with promising results although further validation is required (Table 1). Only a few antilisterial lysins have been assessed in food products and the food matrix and environment have been found to affect the antimicrobial activity (40).

Table 1. Antilisterial lysins reported in literature, key summary and application.

Endolysin	Reported findings	Use[a]	Reference
Ply118	Rapidly lysed all *Listeria* strains tested and against	BC, IC	(45)
Ply500	3 Bacillus species.	BC, IC	
Ply511	Rapidly lysed all *Listeria* strains tested against.	BC, IC	
PlyP35	Determined optimal temperature, NaCl, pH and various ions conditions.	BC, IC	(46)
PlyP40	Lysed *L. monocytogenes* strain and *L. innocua*.	BC, IC	(47)
PlyP825	Inhibited all growth in *L. monocytogenes* strains used.	BC, IC	(48)
PlyPSA	Determined crystallised structure	RMD	(49)
PlyP100	Lysed all *L. monocytogenes*, *Listeria* strains in cheese and a *Bacillus subtilus* strain tested against	BC, BP	(50)
LysZ5	Lysed *L. monocytogenes*, *L. innocua* and *L. welshimeri*; reduced *L. monocytogenes* numbers in soya milk.	BC, BP	(51)
PlyLM	Lysed all *L. monocytogenes* and *L. innocua* strains tested against; digested *L. monocytogenes* biofilms when combined with a protease.	BC	(52)

[a]BC – Biocontrol, IC – Infection control, RMD – Rapid multiplex detection, BP – Bio preservation.

To date there is only one lysin, PlyLM, which has been tested against *L. monocytogenes* biofilms after 100 % susceptibility on planktonic *L. monocytogenes* and *L. innocua* cells was achieved (52). PlyLM reduced the monolayer biofilm to the same level as the application of lysozyme and proteinase K. When used in combination with proteinase K, or both proteinase K and lysozyme, synergistic effects were observed, and the biofilm was effectively digested. However, biofilms were only grown for 24 hours at 37°C and therefore the efficacy of these enzymes under other conditions merits further investigation, for example performance at lower temperatures which are more reflective of those of most FPEs. More research has been undertaken on staphylococcal biofilms, predominantly monospecies biofilms, which have

achieved reductions in biofilm mass. Of interest is their efficacy against persister cells. Persister cells are metabolically inactive sub-populations of cells, which are “super-resistant” to antimicrobial agents such as antibiotics (53, 54). Studies have shown these persister cells occur as a sub-population of bacterial biofilms, and as such can present a significant obstacle to biofilm inactivation by antimicrobials (53, 55). Several studies have shown a promising role for lysins to inactivate persister cells in biofilms (56, 57). The success being reported against staphylococcal biofilms suggests the potential lysins may have against biofilms in a food production context, particularly in targeting *Listeria* biofilms, which are a significant problem in FPEs. Another phage enzyme, extracellular polysaccharide depolymerase, has also been shown to degrade biofilm EPS however it is highly specific to the strains the phage infects (37). A similar approach targeting *L. monocytogenes* in biofilms could also present an alternative control measure.

Competitive Bacterial Species

Competitive exclusion is where one bacterial species competes with another species over resources and/or space in a habitat, successfully reducing the number of cells or excluding that species (58). This competitive exclusion can be the result of the production of antimicrobials such as bacteriocins, organic acids either acting directly against the species it is competing with or acting on the environment altering the pH, or alternatively physically outcompeting other bacterial species for nutrients and/or space and limiting normal survival or proliferation of those competitive species. This strategy is typically categorised into three components: competition, where planktonic cells of both species are co-cultured for a period of time; exclusion, where the antagonistic species are grown to a biofilm cell density prior to the addition of planktonic cells of the target species; or displacement, in which the target species are grown to biofilm cell density prior to addition of planktonic antagonists (59, 60). As biofilms protect microorganisms from chemical cleaners and disinfectants, the use of non-

pathogenic microorganisms may assist sanitation approaches in controlling, preventing or eradicating unwanted species like food borne pathogens.

Competitive exclusion studies typically pit planktonic cells of the antagonist species (i.e. the species which will exert a competitive exclusion effect) against planktonic cells of the target species in a competition assay, grown together for a period of time facilitating biofilm formation. Daneshvar Alavi and Truelstrup Hansen (61) used a short incubation time of 72 h which resulted in a 1-log decrease in *L. monocytogenes* cell density after application of *Serratia proteamaculans.* A similar reduction was also reported by Fox et al (62) of *L. monocytogenes* biofilm cell density after 96 h when grown in co-culture with *Janthinobacterium lividum*. However, greater reductions have been reported when cells were incubated for longer periods with results around log 4.5 and 5.5 on stainless steel coupons and polytetrafluoroethylene respectively (60). Zhao et al (63) also reported higher magnitude reductions of 7.8-log reduction over 28 days at 15°C by two bacterial isolates, *Lactococcus lactis* (*Lc. lactis*) and *Enterococcus durans*. In another experiment performed at 8°C for 28 days four isolates, including the previous two isolates were also capable of reductions around 7-log units. However, the higher reductions reported by Pérez-Ibarreche et al (60) and Zhao et al (63) were produced by lactic acid bacteria (LAB) whose inhibitory activity has been studied extensively for many years, particularly as probiotics (64).

The inhibitory effect of LAB was further explored by Guerrieri et al (65) and Gómez et al (66) as a preformed biofilm preventing *L. monocytogenes* biofilm formation as part of the exclusion strategy. Gómez et al (66) tested a variety of LAB strains and found reductions ranged from 4 to 7-log units over 24 and 48 h however, by 72 h *L. monocytogenes* growth had increased by almost half fold of the control indicating these strains were only capable of exclusion within the first 24-48 h. However, *Lc. lactis* 368 strain was able to completely exclude the growth of *L. monocytogenes* for the entire period, although it should be noted that all experiments were performed at a relatively elevated temperature and as such lower temperatures reflective of

many food production environments require further consideration. In comparison, Guerrieri et al (65) showed the potential of LAB bacteria at refrigeration temperatures with a *Lactobacillus plantarum* (*Lb. plantarum)* strain capable of a 4-log reduction over a 10-day period. Mariani et al (67) used the native biofilm microflora of wooden cheese ripening shelves to achieve a 1 to 2-log reduction over a 12 day period, although this reduction was less than that observed in Guerrieri et al (65) and Gómez et al (66).

The third strategy displacement, as reviewed by Woo and Ahn et al (59) demonstrated the use of planktonic antagonist LAB strains as a post treatment control method targeting *L. monocytogenes* was less effective compared to pre-treatment, although two strains (*Lb. paracasei* and *Lb. rhamnosus*) were capable of a 3-log reduction in *L. monocytogenes* biofilm cell density over 24 h when incubated at 37°C.

While most studies are performed in laboratories, Zhao et al (68) and Zhao et al (69) took the concept of competitive exclusion a step further and looked at its applicability in poultry processing facilities. In a fresh poultry facility, two LAB strains (*Lc. lactis* and *E. durans*) were added to two enzyme-based cleaners and applied as a foam to selected drains four times in the first week, then two times for the following three weeks. Sampling continued for 18 weeks after the last treatment. Most drains experienced significant reductions within the first week after only four applications and all drains maintained lower levels of *Listeria* throughout the sampling period (68). Importantly, two drains reported significant reductions 16 weeks after treatments finished. Similar parameters were applied to the application of the same strains at a ready to eat poultry processing facility. By the end of the first week of application *Listeria* was not detected in five of the six drains with all drains reporting negative results between weeks eight and 13 (69). It should also be noted the strains utilised were known to either possess nisin or other forms of antimicrobials, however, it was not elucidated if the inhibition was the result of the production of antimicrobials.

There have been some encouraging results in the use of LAB against *L. monocytogenes* biofilm cells in laboratory-based experiments (Table 2) however very few have been trialled in actual FPEs, apart from Zhao et al (68) and Zhao et al (69). The results from their two studies have shown promising results as an alternative control method utilising *E. durans* and *Lc. lactis* however, further longitudinal research surrounding the in-facility application is required. In addition, the application of other bacterial species identified in some of the studies mentioned above, for example *J. lividum* and *S. proteamaculans* warrant in-facility testing. However, it should be noted that the LAB strains utilised for in-facility application studies were isolated from the production environment indicating that specific strains may work best in the environment they were isolated from and these strains may vary depending on the food industry.

Houry et al (71) reported the use of bacterial species in a novel biocontrol approach. In the study they identified a subpopulation of bacilli known as bacterial swimmers which were capable of creating transient pores within the biofilm structure. By pre-treating *Staphylococcus aureus* biofilms with bacterial swimmers, which also produced an anti-staphylococcal bactericide, they achieved a greater inactivation of *S. aureus* in biofilm by facilitating access of toxic substances in the environment into the biofilm.

Table 2. Bacterial species active against *L. monocytogenes* and purported mode of action.

Bacterial Species	Mode of Action	Studies
S. proteamaculans	Sanchez et al identified a bacteriocin-like substance was produced at low temperatures capable of inhibiting *L. monocytogenes*. Inhibition was suggested to be the result of Jameson effect.	(61, 70)
J. lividum	Specific strain utilised not tested for antimicrobial compounds. *J. lividum* are reported to have antibacterial compounds capable of inhibiting Gram-positive bacteria.	(62)
Lc. lactis	Neither of the studies by Zhao et al. tested for production of a bacteriocin, however this species has previously been reported to produce nisin.	(63, 68, 69)
E. durans	Neither of the studies tested for the bacteriocin, however this species has previously been reported to produce enterocin.	(63, 68, 69)
Lb. plantarum 396/1	Inhibition was attributed to production of an organic acid.	(65)
Lb. paracasei	May be the result of competition for sites and resources. As a probiotic strain it may produce bacteriocin, organic acid or hydrogen peroxide.	(59)
Lb. rhamnosus	May be the result of competition for sites and resources. As a probiotic strain it may produce bacteriocin, organic acid or hydrogen peroxide. A previous study isolated an antilisterial bacteriocin from this species.	(59)
Lb. sakei	Bacteriocin producing strain.	(60)
LAB – Lc. lactis 368, Lb. helveticus 354, Lb. casei 40, W. viridescens 113	Not identified as bacteriocin-producing strains. May be result of biosurfactants, or exclusion by trapping (killing cells embedded in biofilm).	(66)
Native microbial flora of cheese ripening wooden shelves.	Established biofilms on active cheese ripening wooden shelves were used. Inhibition may have been the result of competition for sites and nutrients.	(67)

Bacteriocins

An important component of the competitive survival strategy of bacteria is the production of antimicrobial products. One group of ribosomally synthesized antimicrobials are the heat stable peptides known as bacteriocins (72-74). It has been suggested that most bacteria produce at least one bacteriocin and LAB are known to be prolific producers (72). Most bacteriocins have a narrow spectrum of activity: that is they are active against the same species that produces them but the producer is immune to them, while some have a broad spectrum of activity acting on members within the same genus as well as other genera and species (72). The mode of activity varies depending on the particular class of bacteriocin and can include pore formation, or inhibition of key cellular processes such as peptidoglycan production, DNA replication, mRNA or protein synthesis, to name a few (72). There are two main groups: Class I (also known as lantibiotics): peptides that undergo post-translational changes; and Class II, which do not (75). Among the most well-characterised and successful bacteriocins to date is nisin, a Class I bacteriocin from *Lc. lactis* which has been approved for use in food as a preservative/additive by the World Health Organisation, European Union and the United States Food and Drug Authority (72). A great deal of research has gone into identifying more bacteriocins active against *L. monocytogenes* planktonic cells and biofilms, an important arena as nisin resistance is slowly being reported.

Most studies can be classified into two groups based upon how the bacteriocin is applied: either as whole bacterial cells known or suspected of bacteriocin production, or alternatively the bacteriocin extract itself, applied either as a crude or semi-purified product. Their utility against preformed *L. monocytogenes* biofilms of varying times has been the subject of numerous studies, with some reporting promising results. For example, Gómez et al (66) assessed *Lc. lactis*, *Lb. sakei* and *Lb. curvatus*, all known to produce nisin Z, sakacine A and sakacine P respectively, against 48 h preformed biofilms. *Lb. sakei* and *Lb. curvatus* were capable of complete inactivation over 72 h whereas the two *Lc. lactis* strains provided a 6-log

reduction by the end of the test period. Winkelströter et al (74) however were unable to produce results of the similar magnitude when *L. monocytogenes* was co-cultured with *Lb. paraplantarum,* only achieving 2-log inactivation at 24 and 48 h before decreasing by 72 h. Guerrieri et al (65) took an alternative approach and reported *Lb. plantarum* and *Enterococcus casseliflavus* were able to inactivate *L. monocytogenes* 7 day preformed biofilms by 3.9 and 3.7-logs over a 10 day period. Importantly the results could be associated with bacteriocin production, as no changes to the pH were observed.

Another technique is extracting the bacteriocin in the form of cell-free supernatant (CFS), as a crude bacteriocin fermentate or semi purifying the product. The antimicrobial activity of CFS has shown mixed success in co-inoculation studies to prevent the formation of biofilms by *L. monocytogenes*, with Camargo et al (76) reporting significant reductions after 24 h, whereas Bolocan et al (77) only observed between 1.6 and 3.6-log CFU/cm^2 reduction after 72 h depending on the media used. In the latter study however, the CFS extract which produced the highest reduction was from an isolate known to also produce an organic acid which was not removed and therefore this result may not be associated solely to the antimicrobial activity of the bacteriocin. When Camargo (76) applied the CFS to 24 h preformed biofilms for 2.5 h they found biofilm formation continued in some isolates.

Other researchers have compared the two methods, bacterial cells and extracts again with varying results. Garcia-Almendárez et al (78) analysis on four-day preformed biofilms demonstrated a crude bacteriocin fermentate from *Lc. lactis* known to produce nisin A was capable of a 2.7-log reduction over 24 h. However, a greater reduction over 5-logs was achieved when the *Lc. lactis* was applied for 6 h then rinsed and placed in a desiccator for five days. Whereas Winkelströter et al (79) co-inoculated *L. monocytogenes* with *Lb. sakei* or its CFS and found any decreases observed in the first 24 h were diminished with time, as results at 48 h were comparable to the pure culture levels. A promising approach by Pérez-Ibarreche et al (60) involved the supplementation of *Lb. sakei* cells with a semi purified bacteriocin for 6

h, which resulted in a two-fold reduction in *L. monocytogenes* numbers on the stainless steel surface, or an additional 1-log reduction on polytetrafluoroethylene.

As mentioned previously the bacteriocin nisin has been approved for commercial purposes and has paved the way as an alternative biocontrol method. Research into bacteriocins have been performed with comparable results to the other non-commercial bacteriocins discussed above. Minei et al (80) found nisin was capable of inhibiting *L. monocytogenes* biofilm formation for 9 h on stainless steel coupons, and although cell growth did recommence after this time, a 3.5-log inactivation was still maintained by 48 h. On the other hand, Henriques and Fraqueza (81) shortened the treatment time to 5 min and even at the highest concentration no activity was recorded, although activity was defined as a ≥ 5-log decrease.

From the above, it is obvious that results vary significantly depending on if bacteriocin producing bacterial cells or the bacteriocin extracts are used. Results from bacteriocin extracts can be correlated to the antimicrobial action of the bacteriocin with greater certainty however additional analysis is required particularly when whole cells are used to help ensure that the measured inhibition is not the result of competitive exclusion or the production of other antimicrobials such as organic acids. The co-inoculation and preformed biofilm studies reflect the ability of the bacteriocin to either prevent the formation or affect the removal of established biofilms in the FPE, however the length of time the biofilms are grown for prior to the bacteriocin being applied also affects the antimicrobial activity as mature biofilms may provide better resistance. Although several studies show promising results most require additional analysis at temperatures and other environmental conditions mirroring the FPE to identify potential candidates suitable for further testing. With the potential resistance to nisin arising, the identification of other bacteriocins is essential. In addition, the application of synergistic antimicrobials to further combat the development of resistance should be considered.

Plant derived antimicrobial products – Essential Oils

An alternative to the use of chemicals, microorganisms or their derivatives is the use of plant derived antimicrobial products such as essential oils (EOs). Herbs and spices are commonly known to exhibit antimicrobial activity and have been used by various cultures for flavouring, as a food preservative or for medicinal purposes. EOs play a key role in protecting plants from bacteria, fungi, viruses, insects and animals (82). Traditional distillation, cold press/expressing, solvent extractions and enfleurage methods have been used to extract EOs from plant-derived materials; more recently modern techniques including microwave or ultra sound assisted extraction, pressurised extractions and super critical fluid extraction have been used to obtain EOs from a variety of plant sources (including roots, wood, bark, twigs, leaves, seeds, buds, flowers and fruits). However, the constituents and compositions of EOs vary significantly from high concentrations to trace amounts based upon the plant part, plant age and extraction method used, in turn influencing their antimicrobial activity (82-85). Key molecules in EOs with the most effective antibacterial activity are typically from aldehyde and phenol chemical classes which includes compounds such as cinnamaldehyde, carvacrol, eugenol or thymol (82, 86). Essential oils are able to permeabilise the cell membrane resulting in the leakage of ions or other cell content, and may also disrupt key genetic functions and/or cellular components like proteins, polysaccharides, phospholipids, fatty acids and essential enzymes due to the lipophilic nature of EOs (82, 86-88).

While there are thousands of essential oils described, it is reported around 300 of these have generally recognised as safe approval and are used commercially for flavouring or fragrance, however more detailed information is required for their use as a biocontrol agent (83, 89). Most research surrounding the antimicrobial activity of EOs focuses on their effects on planktonic cells of food spoilage and pathogenic bacteria either in standard laboratory conditions or their application on food items. This application on food as a biocide has major limitations as higher concentrations are required potentially interfering with the sensory attributes of the food (89,

90). In addition, some components of food items, mainly fats, proteins, carbohydrates, water, salt, antioxidants, pH and other preservatives or additives used may impact upon the activity of the EOs (82). Further research is required to understand the impact EOs have on bacterial pathogens and in particular their ability to prevent or eradicate biofilms in FPEs. Some research is occurring within this space however, there is limited research against *L. monocytogenes* biofilms with a few studies looking at the extracted EOs, the active components of specific EOs, or altering the EO chemical composition. de Oliveira et al (87) assessed the EOs from fresh citronella (*Cymbopogon nardus*) and lemongrass (*Cymbopogon citratus*) leaves applied alone or in combination, however it was the Citronella EO which demonstrated the highest reductions against both the 3 h and 240 h preformed biofilms with complete reduction after 60 mins of application. Similar results reported in another study by de Oliveira et al (88) found 2 % (vol/vol) Chinese cinnamon extract (*Cinnamomum cassia*) was capable of reducing a 48 h preformed biofilm to below the detection limit (2.84-log CFU/cm^2) after 20 mins however both of these studies applied the EOs at temperatures above 20 ºC.

Essential oils contain a mixture of major and minor molecules responsible for their antimicrobial activity with some of the major components being explored further. The active components of clove (eugenol) and spearmint (carvone) EOs were tested on a 6 h preformed *L. monocytogenes* biofilm but were found to increase biofilm mass by Leonard et al (91). Citral and nerol, in contrast, both major components from lemongrass (*C. citratus*) and *Lippia rehmannii* (nerol only), displayed a similar reduction as the positive control ciprofloxacin.

Additional microbial species can also impact upon the activity of the EO or active component. For example, Leonard et al (91) study as mentioned above was on *L. monocytogenes* monospecies biofilms and reported a mixture of results among the EO and the various active components tested. Whereas de Oliveira et al (92) looked at the activity of Chinese cinnamon and its active component, Cinnamaldehyde on a mixed biofilm of *L. monocytogenes* and enteropathogenic *E. coli* on stainless steel coupons dipped in reconstituted whole milk. The

EO and cinnamaldehyde were both capable of reducing the mixed biofilm to below the detection limit of 2.84-log CFU/cm^2 whereas the EO and active components only provided reductions just over 2-logs on the *L. monocytogenes* biofilm. Chorianopoulos et al (90) examined the EO and hydrosol (by-product of the steam distillation) of *Satureja thymbra* (Pink Savory) and showed similar results when grown in a mixed biofilm with a food borne pathogen (*L. monocytogenes*, *Salmonella enterica*) and a spoilage bacterium (*Pseudomonas putida*). It was noted that the optimised application time was 60 mins and any increase in time provided no additional reduction. The impact other microbial players may have on the activity of EOs requires further exploration in order to gain insights into the various relationships at play.

A common problem for the use of EOs as a biocontrol method on food products is the associated impacts on taste at concentrations required for appropriate antimicrobial effect. A process to concentrate the EOs for application at a lower volume with the same potentially high antimicrobial activity may be required in the case of some EOs. Krogsgård Nielsen et al (93) looked at emulsifying and encapsulating isoeugenol oil to increase the antimicrobial effectiveness at a smaller volume with the addition of electrostatic forces to attract negatively charged bacteria to positively charged EOs. Although the concept of emulsification and encapsulation sounds promising the minimal biofilm eradication concentration (MBEC) for the coated and uncoated emulsified products were only half a log lower than the pure isoeugenol when tested in standard laboratory medium at three temperatures (6, 12 and 25 °C) and no difference was observed in carrot juice. This observation requires further exploration as the reductions in the MBEC did not correlate to observations under confocal microscopy. Of note was the morphological changes observed in the mixed biofilms of *Pseudomonas fluorescens* and *S. aureus* from uniform layers to clusters of numerous cells, which requires further research to determine if there are any implications.

As mentioned previously the use of EOs at concentrations to exhibit sufficient antimicrobial activity has the potential to impart undesirable flavour and when applied in a FPE may also

result in an excessive sensorial impact. In addition, the interactions of EOs with components of the food matrix from food debris may also impact on the applicability of EOs in food environments. Only a few studies have investigated the application of EOs to disrupt or prevent the formation of biofilms. Further research on parameters specific to industry will allow a better decision on the application of EOs as an alternative or supplementary biocontrol method.

Concluding remarks

While current sanitation processes are effective against planktonic cells the potential for tolerant strains to increase due to interactions at subinhibitory levels and the potential reliance on them as antimicrobials, as the case in the health industry, is a cause for concern. The ability to eradicate established biofilms and prevent new biofilms from being formed is a challenging task which food production managers are charged with, as biofilms can present increased food safety risks. A useful tool in understanding the microbial community is metagenomics analysis of the FPE. By understanding the FPE microbiome valuable information can be gained regarding persistence or transience of strains. This facilitates source tracking of persistent strains, can identify other microbial species that may provide either a positive or negative effect on the target strain and can identify strains surviving the disinfection processes (94, 95). From this information the appropriate biocontrol method can then be determined. There have been some significant advances in the development of biocontrol methods, particularly bacteriophages that have progressed to commercial products with the results of some studies validating their progression to commercialisation. The use of competitive bacterial species has also showed some promising results with the concept of utilising antagonist strains isolated from the production environment providing individualised treatment options. Bacteriocins and endolysins have also shown their ability to significantly reduce established biofilms however they typically require some form of purification process to achieve these results. The sensory implications of essential oils at concentrations required to

exert antimicrobial effects is a limiting factor in their use as a sole biocontrol method and therefore they may find more appropriate utility as a supplementary method targeting non-food contact surfaces. However, like all biocontrol methods efficacy can be impacted by a variety of factors including temperature or time the control method was applied for, the use of one species or multiple species biofilms, biofilm growth method, or surface matrix composition. Standardisation in the assessment of novel biocontrol methods against biofilms is required, in addition to assessment under conditions reflective of FPEs before appropriate comparisons can be made.

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Chapter 6

General Discussion

Listeria monocytogenes is an important food borne pathogen; it can cause foodborne illness in at risk population groups with the main vector of transmission being ready to eat food products. Entry into the FPE can occur through raw ingredients, and the bacterium may be further disseminated throughout the facility by employee movement allowing isolates to colonise and potentially persist within the FPE over prolonged periods. This doctoral research project sought to improve our understanding of dynamics and mechanisms underlying *L. monocytogenes*' ability to survive and persist within the FPE by looking at various colonisation factors potentially at play.

A key component of colonisation and persistence within the FPE is a bacterium's ability to form biofilms. While there has been substantial research in understanding *L. monocytogenes* ability to form biofilms in conditions relevant to human infection, there is limited information considering conditions applicable to the FPE, partially due to the difficulty conducting controlled experiments under conditions that replicate the FPE (1). Therefore, Chapter 2 focused on developing a high throughput model system capable of assessing biofilm formation using conditions such as low temperature, low nutrition and a stainless-steel surface, which are characteristic of food processing facilities. This method provided fast and efficient results, facilitated scaling up to large numbers of isolates to measure their ability to form biofilms and identified strains of interest for further research.

In Chapter 3, this model system was used to assess the biofilm formation of 52 isolates leading to the identification of five fast attaching isolates and five slow attaching isolates which were further assessed to identify differences between fast and slow forming isolates against a selection of colonisation dynamics. Quorum sensing has been shown to be involved in various

stages of biofilm formation and dispersion (2), with a common signalling peptide in low GC Gram-positive bacteria being the *agr* system autoinducing propeptide. Rieu and colleagues (3) showed at 25°C *agr* expression was involved in early stage biofilm formation and possibly played a role in the initial attachment process, with no further part played after attachment. In this study we assessed the role *agr* plays in biofilm formation at 24 and 48 h in low temperature and nutrition conditions. While *agr* expression was shown to be upregulated at two h and decreased by 24 h in the study by Rieu et al, in this study *agrD* was observed to be upregulated at 24 h on coupons indicating that the conditions utilised in this study may extend the early stages of biofilm development to 24 h. The expression of *agrD* in biofilm cells compared to planktonic cells are in line with the observations of Rieu et al and suggest that even at the lower temperature and low nutrient conditions used in this study, the *agr* system is upregulated in the switch to attachment and early biofilm formation. This suggests the *agr* system may have a universal role in *L. monocytogenes* initial attachment and early biofilm formation.

The use of GWAS in clinical disease analysis has seen great success; however, the application of GWAS in microbial studies has been more limited, with success only being present in microbial studies where there is a clear set of genes linked to the phenotype (4). Contrary to Lee's study (5), in which they identified numerous genes associated with biofilm formation in the nutrient condition they used, this study did not find any genes of interest utilising a core SNP approach. While both studies looked at compounding FPE elements, Lee's study compared biofilm formation at suboptimal and optimal growth therefore allowing a more clear-cut identification of purported genes involved, compared to the ability to form biofilms between fast and slow biofilm formers in this study.

While *L. monocytogenes* is not known to be a strong biofilm producer like *Salmonella* strains, which possess genes for extracellular polymeric substances (EPS) like cellulose and curli fimbriae, responsible for the rough dry and red morphotype and demonstrated to be involved in biofilm formation (6), there are conflicting reports on the production of EPS in *L.*

monocytogenes. A recent study by Chen et al (7) showed *L. monocytogenes* is capable of producing EPS with a novel composition that binds Congo red. Isolates displaying a pink phenotype were indicative of some EPS production, however this phenotype was not associated exclusively with the fast biofilm formers suggesting EPS production is strain-specific.

Lastly, Chapter 3 also looked at differences in transcriptome expression between fast and slow isolates in the low temperate and low nutrient conditions. A range of metabolic and transport systems, the cobalamin dependent gene cluster, and prophage related genes were overexpressed in the fast isolates, suggesting nutrient scavenging and utilisation of alternative metabolic substrates are essential for *L. monocytogenes* cells undergoing stress conditions to maintain necessary energy systems for continued survival and proliferation. In addition, various regulators were differentially expressed, indicating the complex response *L. monocytogenes* utilises in reacting to various stress related conditions. In this study *recA* was upregulated in three isolates and is responsible for activation of the SOS response during stressful conditions. In addition to *recA*, other regulators including *lexA*, *degU*, *mogR*, *fur*, *sigB* and *ctsR* were also differentially expressed and all play a varied role in stress response and a potential role in biofilm formation. Overall, the results from Chapter 3 demonstrate that *L. monocytogenes* mounts a diverse stress response under conditions reflective of the FPE.

Whole genome sequencing and genomic analysis of bacterial genomes have increased substantially with the advances and affordability of sequencing technologies. With the generation of high-quality complete genomes of core reference strains of *L. monocytogenes*, such as EGD-e, ScottA and F2365, substantial progress in elucidating the genomic landscape of this bacterium has been established. Further genomic analysis of additional *L. monocytogenes* strains is required to facilitate insights into genomic changes, environmental, virulence and resistance adaptability and advanced evolutionary understandings in a global context, incorporating diverse ecosystems. The Australian *L. monocytogenes* population

structure has previously been eluded to (8), however, the genomic and phenotypic analysis of *L. monocytogenes* isolates from Australia, which contributed to 63 % of the isolates utilised in this study, are lacking in the literature. Moreover, larger scale comparative genomic analyses correlated to phenotypic screening of food-associated isolates has been limited. Chapter 4 focused on phenotypic and genomic analysis of *L. monocytogenes* strains utilised in the high screening assay of Chapter 3 to enhance the current knowledge of *L. monocytogenes* fitness and potential to survive within the FPE, as well as virulence potential and control of strains in both FPE and clinical paradigms.

The presence of virulence determinants in *L. monocytogenes* is indicative of the ability to cause disease. Recent studies by Maury et al (9) have suggested hypervirulence is present in some *L. monocytogenes* CCs, for example CC1, CC2, CC4 and CC6, as a result of certain virulent determinants being present in the genome. In this study, genomic regions associated with hypervirulence were identified in three CC1 and interestingly two CC3 strains, which also contained SSI-1, the latter of which was not noted as a hypervirulent CC by Maury et al.

The presence of *inlA* mutations has previously predominately been associated with CCs over-represented in food systems, like CC9, CC121, CC155 and CC321. The presence of these mutations is linked to reduced invasion capability in the mammalian host. In addition, the presence of SSIs has been shown to increase survival of isolates. In this study, the presence of presumed hypovirulent strains was slightly more common than hypervirulent strains based upon CC association and hypervirulence/hypovirulence determinants, however further characterisation of significantly more isolates is required to determine the hypervirulent/hypovirulent landscape in Australia and Ireland.

Chapter 4 also examined the potential mechanisms that could contribute to survival in the FPE, with the presence of cadmium and disinfectant resistance genes determined to assess their concordance with the phenotypic tests. All isolates which displayed increased levels of

cadmium resistance were correlated to the presence of six cadmium gene cassettes present within the *Listeria* genus; interestingly, one of these represented a novel *cadA* variant, termed *cadA7*. Resistance to benzalkonium chloride (≥ 5 µg/mL) was also correlated with the presence of a disinfectant resistance gene in all but two isolates. Interestingly, suboptimal concentrations of BC have been linked with increased biofilm formation (10); furthermore, the presence of cadmium or disinfectant resistance genes has been linked with co-resistance (11). Therefore, the presence of disinfectant or heavy metal resistance determinants in *L. monocytogenes* strains is likely to lead to increased fitness within the FPE, and the selection of stress resistant variants. The novel cadmium gene, *cadA7*, identified on a transposon which may have been horizontally acquired from *Enterococcus*, is an important discovery reiterating the need for continuous analysis of the genetic potential and adaptability of *Listeria*. Another novel insert was identified in strain 8115 in this study in the hypervariable region known to house LGI1. This novel insert shared similarity to Tn*916*, however additional research is required to determine the function of this Tn*916* variant in 8115, and whether it provides an advantage within the FPE or during pathogenesis. Notably, this mobile genetic element contained a novel gene, predicted to encode an efflux pump system protein. All isolates in this study were sensitive to the five clinically relevant antibiotics tested supporting the successful treatment of listeriosis by these antibiotics.

Increased tolerance to disinfectants, heavy metals and food processing stresses warrants additional strategies to help control *L. monocytogenes* within the FPE (Chapter 5). Utilising beneficial microbes, their antimicrobial products and plant derived products has the potential to offer an alternative, synergist or supplementary mitigation method. Biocontrol options include bacteriophages and their endolysins, competitive bacterial species and their bacteriocins and plant derived essential oils. For food processing hygiene managers to make an informed decision, research into these biocontrol options needs to reflect the multiple factors at play within the FPE. These include temperature, the various surfaces present, food matrix and current microbial context of the facility. Good success has been shown against

planktonic cells, particularly bacteriophages which have had commercial success as a food preservative. However, further research is required on interventions targeting biofilms at various stages. Initial reports have indicated promising results across all biocontrol options; however, some draw backs have been identified; notably surrounding the utilisation of lysins and bacteriocins, which generally requires additional research to further elucidate and validate efficacy. In addition, understanding the microbial context of the FPE and utilising facility-specific competitive bacterial species, or their antimicrobial products, can provide increase control and individualised treatment of *L. monocytogenes*. However, this typically requires an initial financial outlay by the food producer to gain this insight, which may limit small processing facilities from being able to access the most appropriate and effective biocontrol system for their facility. Nonetheless, the utilisation of biocontrol methods is a novel approach which warrants further attention, particularly surrounding their effectiveness against multi-species biofilms, conditions reflective of the FPE and in-facility application.

While this study has provided beneficial insight into our understanding surrounding the colonisation dynamics and potential of *L. monocytogenes*, it is not without its limitations. Bacteria can exist in single or multispecies biofilms and within the biofilm cells can be in various metabolic states therefore further research mining a wider panel of isolates, and in more complex communities is needed. Furthermore, to provide a more comprehensive understanding, multiple factors, like those in this study, should be analysed individually and in combination to provide a more refined understanding of the influence of specific drivers. Chapter 4 identified novel genes and inserts of interest, however, it was not an exhaustive analysis of phenotypic and genotypic traits associated with FPE survival, virulence and therapeutic potential, and therefore additional analysis is required to provide a comprehensive outlook at the current state of *L. monocytogenes* strains isolated from the FPE. Furthermore, this study did not characterise invasion and pathogenicity in model systems, to elucidate the potential associated roles of the novel inserts and genes identified, along with the general

invasion landscape of the isolates. In particular, further such interrogation of isolates with *inlA* mutations or hypervirulent determinants should be subject to further research.

In conclusion, the genomic and phenotypic analyses undertaken in this study enhance the current knowledge on *L. monocytogenes* strains ability to colonise the FPE. Through the development of a high throughput screening method, we were able to characterise the variability in the capacity of different strains to attach and form biofilm at conditions reflective of the FPE. Taken all together, the *L. monocytogenes* strains in this research showed a dynamic cellular response characterised by mechanisms likely to aid survival and persist within the FPE. This work demonstrates the importance of continued surveillance of *L. monocytogenes* in the FPE, and the need for further understanding of its ability to efficiently colonise the FPE. The ongoing development of alternative control methods is reinforced by the widespread disinfectant and stress tolerance mechanisms disseminated among food-associated strains noted in this research. *L. monocytogenes*' ability to colonise and survive within the FPE appears to be a complex strain-specific phenomenon, requiring further research throughout the food industry and wider academic community.

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Appendix A

Chapter 3 - Colonisation dynamics of *Listeria monocytogenes* strains isolated from food production environments

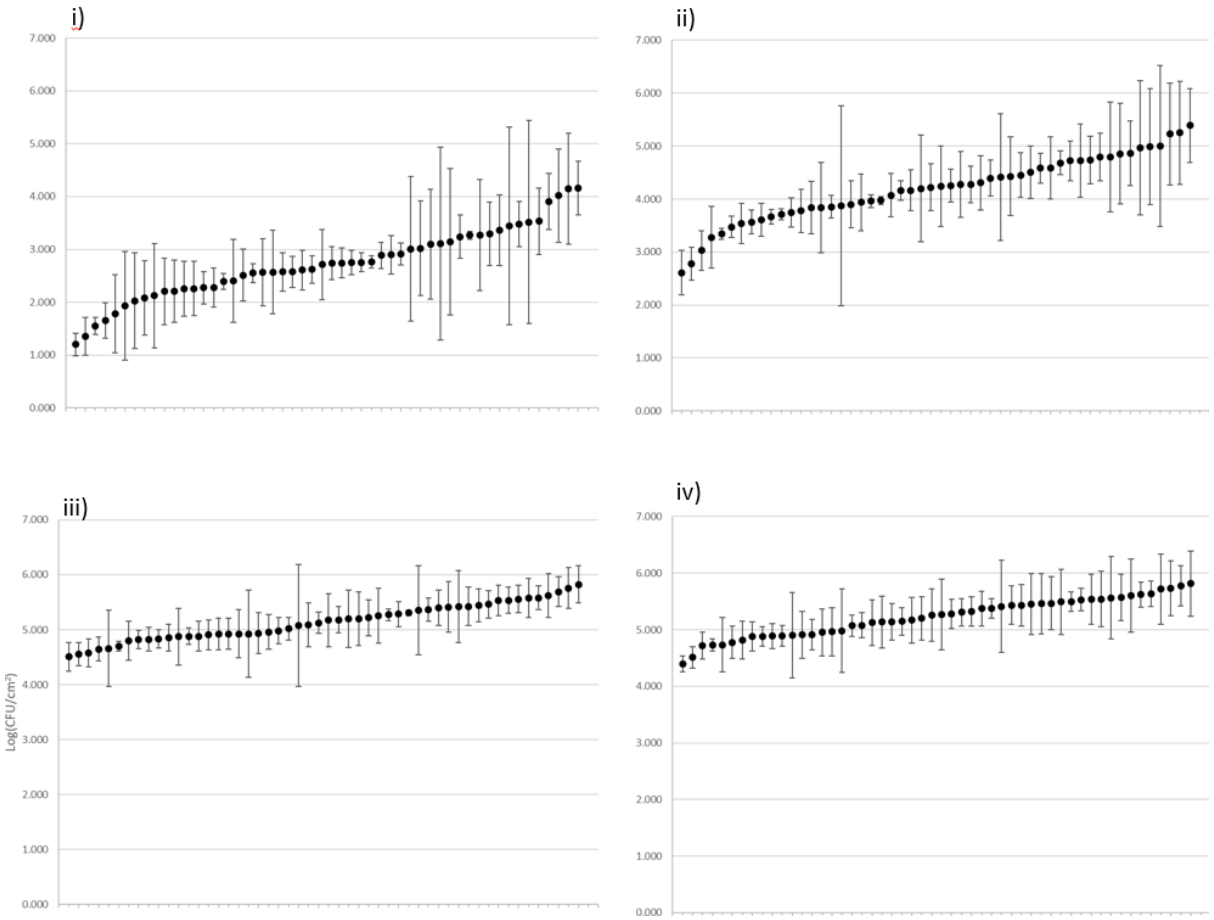
Supplementary Table A1. Isolate information.

Isolate	MLST ST	Serotype	Year isolated	Description	Lineage	CC	Biofilm phenotype[#]	Genebank Accession #
7425	121	1/2a	2011	Environmental - Meat	II	CC121	Fast	
7452	155	1/2a, 3a	2009	Dairy - Milk	II	CC155		NAUM00000000
7453	101	1/2a, 3a	2009	Dairy - Cheese	II	CC101	Fast	NAUH00000000
7456	155	1/2a, 3a	2010	Dairy - Milk	II	CC155	Fast	NAUL00000000
7461	3	1/2b, 3b	2010	Dairy - Milk	I	CC3		
7475	121	1/2a, 3a	2011	Dairy - Cheese	II	CC121		NAUF00000000
7479	1	4b, 4d, 4e	2012	Dairy - Cheese	I	CC1		
7484	101	1/2a, 3a	2012	Dairy - Cheese	II	CC101		NAUG00000000
7488	204	1/2a, 3a	2012	Dairy - Cheese	II	CC204	Slow	LXQX00000000
7495	121	1/2a, 3a	2012	Dairy - Cheese	II	CC121		NAUE00000000
7514	3	1/2b, 3b	1998	Vegetable	I	CC3	Slow	
7523	1	4b, 4d, 4e	1988	Meat - Beef	I	CC1		NAVA00000000
7530	2	4b, 4d, 4e	1998	Dairy - Ice cream	I	CC2		
7533	155	1/2a	2007	Environmental - Meat	II	CC155		
7535	9	1/2c	2007	Environmental - Meat	II	CC9		LJPE01000000
7536	1	4b, 4d, 4e	2009	Environmental - Dairy	I	CC1	Slow	LJPF01000000
7538	2	4b, 4d, 4e	2009	Environmental - Dairy	I	CC2	Slow	
7540	7	1/2a, 3a	2011	Mixed Food	II	CC7		
7544	204	1/2a, 3a	2006	Environmental - Dairy	II	CC204		LXQZ00000000
7545	2	4b, 4d, 4e	2008	Mixed Food	I	CC2	Fast	
7546	12	1/2a, 3a	2009	Environmental - Dairy	II	CC7		

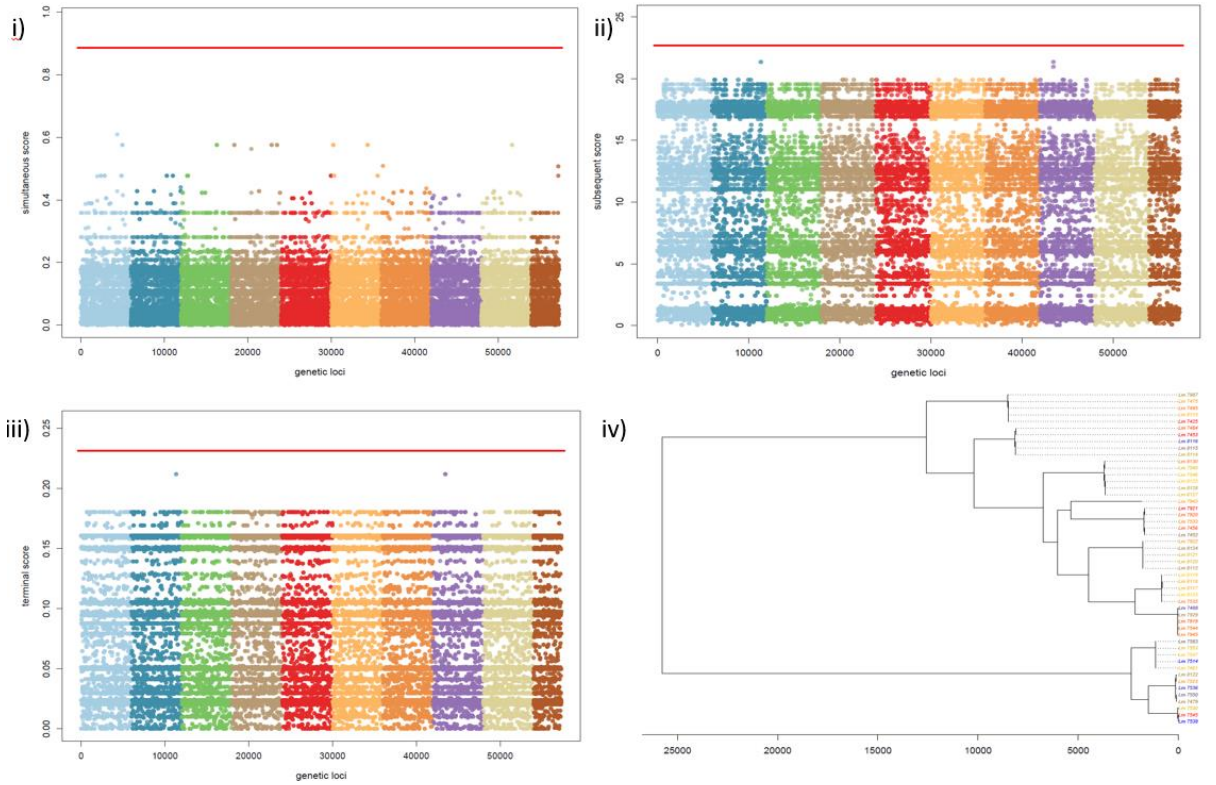
7547	3	1/2b, 3b	2009	Dairy - Cream	I	CC3		
7550	1	4b, 4d, 4e	2013	Dairy - Cheese	I	CC1		
7553	3	1/2b	2007	Environmental - Meat	I	CC3		
7583	3	1/2b, 3b	2007	Environmental - Dairy	I	CC3		NAUZ00000000
7919	204	1/2a	2015	Meat - Boots	II	CC204		LXRA00000000
7920	155	1/2a	2015	Meat - Boots	II	CC155		
7921	155	1/2a	2015	Meat - Boots	II	CC155	Fast	
7922	8	1/2a	2015	Meat - RTE	II	CC8		
7929	204	1/2a, 3a	2015	Meat - Raw Ingredient	II	CC204		LXRB00000000
7943	321	1/2a, 3a	2015	Meat - RTE	II	CC321		
7945	204	1/2a or 3a	2015	Meat - RTE	II	CC204		LXRC00000000
7987	121	1/2a	2016	Meat	II	CC121		
8112	8	1/2a,3a	2010	Meat	II	CC8		SRR6457844
8113	121	1/2a,3a	2009	Environment - Equipment	II	CC121		SRR6457840
8114	101	1/2a,3a	2009	Vegetable	II	CC101		SRR6457839
8115	101	1/2a,3a	2009	Vegetable	II	CC101		SRR6457842
8116	101	1/2a,3a	2009	Vegetable	II	CC101	Slow	SRR6457841
8117	9	1/2c,3c	2010	Meat	II	CC9		SRR6457815
8118	9	1/2c,3c	2010	Meat	II	CC9		SRR6457814
8119	9	1/2c,3c	2010	Meat	II	CC9		SRR6457813
8120	8	1/2a,3a	2010	Meat	II	CC8		SRR6457836
8121	8	1/2a,3a	2011	Meat	II	CC8		SRR6457831
8122	1	4b,4d,4e	2011	Meat	I	CC1		SRR6457825
8123	9	1/2c,3c	2011	Vegetable	II	CC9		SRR6457828
8124	8	1/2a,3a	2011	Environment - Floor	II	CC8		SRR6457823
8125	7	1/2a,3a	2011	Meat	II	CC7		SRR6457820

8126	321	1/2a,3a	2011	Environment - Floor	II	CC321	SRR6457864
8127	7	1/2a,3a	2011	Meat	II	CC7	SRR6457859
8128	7	1/2a,3a	2011	Meat	II	CC7	SRR6457868
8129	9	4b,4d,4e	2011	Environment - Wall	II	CC9	SRR6457857
8130	7	1/2a,3a	2012	Environment - Floor	II	CC7	SRR6457877

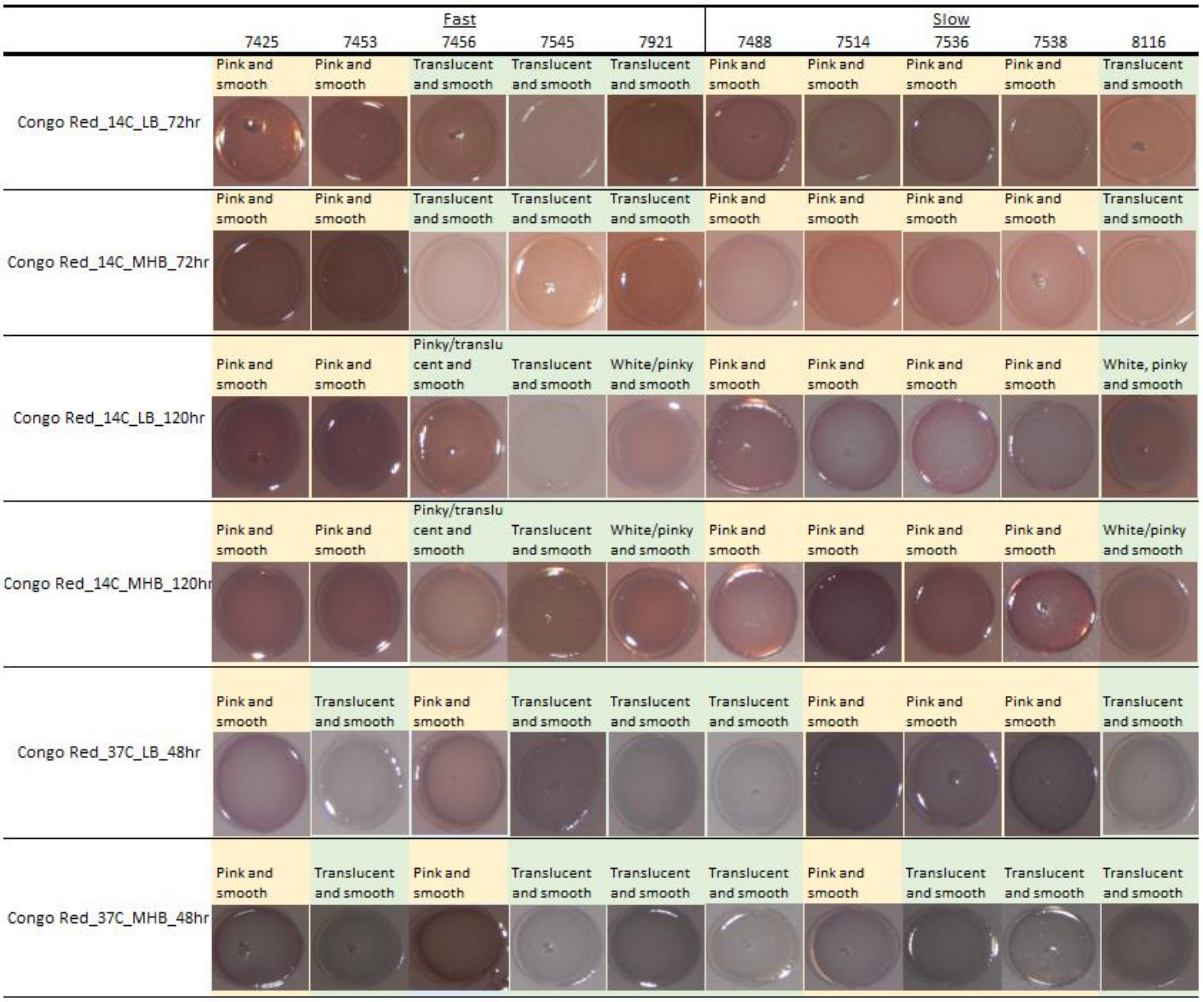
Only scored the B10 group, all others displayed an intermediate biofilm phenotype.



Supplementary Figure A1. Comparison of biofilm formation by 52 *L. monocytogenes* isolates at 14°C in dBHI on stainless coupons at i) 24 h, ii) 48 h, iii) 72 h and iv) 96 h. Biofilm densities (log_{10} CFU/cm^2) were determined every 24 h by standard plate count. Data points represent the average of two biological experiments with two technical replicates, with error bars showing standard deviation. All 52 isolates are displayed on the x axis in numerical order based upon average biofilm density from lowest to highest.



Supplementary Figure A2. treeWAS tests of association. Red line is significance level. i) simultaneous score; ii) subsequent score; iii) terminal score; iv) phylogenetic tree based upon clonal complex, isolates label colours include blue (slow biofilm formers) and red (fast biofilm formers).



Supplementary Figure A3. **EPS production by the B10 isolates on LB or Muller Hinton agar supplemented with 40 µg/mL Congo Red and 20 µg/mL Coomassie Brilliant Blue grown at 14 or 37°C for 48, 72 or 120 h.** Pink phenotype indicative of intermediate ability to produce EPS and translucent phenotype suggestive of no EPS production.

Supplementary Table A2. List of all differentially expressed genes.

This is a separate attachment.

Supplementary Table A3. Summary of the total 494 differentially expressed *L. monocytogenes* genes within the individual and the ST comparison at 24 and 48 h based up their clusters of orthologous groups*.

		7453#		7545#		8116#		ST101_24hr^		ST101_48hr^	
		24 hr	48 hr	24 hr	48 hr	24 hr	48 hr	Up	Down	Up	Down
		CELLULAR PROCESSES AND SIGNALING									
Cell cycle control, cell division, chromosome partitioning	D	3	1	1	0	0	0	0	0	0	0
Cell wall/membrane/envelope biogenesis	M	17	2	11	0	0	0	0	0	0	0
Cell motility	N	4	0	2	0	0	0	0	0	0	0
Post-translational modification, protein turnover, and chaperone	O	2	2	2	0	0	0	0	0	0	0
Signal transduction mechanisms	T	13	0	2	0	0	0	0	0	0	0
Intracellular trafficking, secretion, and vesicular transport	U	4	0	2	1	0	0	0	0	0	0
Defense mechanisms	V	5	2	0	1	0	0	0	0	0	1
Extracellular structures	W	0	0	0	0	0	0	0	0	0	0
Nuclear structure	Y	0	0	0	0	0	0	0	0	0	0
Cytoskeleton	Z	0	0	0	0	0	0	0	0	0	0
		INFORMATION STORAGE AND PROCESSING									
RNA processing and modification	A	0	0	0	0	0	0	0	0	0	0
Chromatin structure and dynamics	B	0	0	0	0	0	0	0	0	0	0
Translation, ribosomal structure and biogenesis	J	22	5	14	10	4	0	3	0	0	0
Transcription	K	16	8	4	2	2	0	0	0	0	1
Replication, recombination and repair	L	10	3	4	1	0	0	0	0	0	0
		METABOLISM									
Energy production and conversion	C	11	6	1	2	0	0	0	0	0	0
Amino acid transport and metabolism	E	14	7	4	1	0	0	0	0	0	0
Nucleotide transport and metabolism	F	8	4	2	2	0	0	0	0	0	3
Carbohydrate transport and metabolism	G	34	12	7	2	0	0	0	0	0	0
Coenzyme transport and metabolism	H	8	3	1	0	0	0	0	0	0	0
Lipid transport and metabolism	I	3	6	1	0	1	0	0	0	0	0
Inorganic ion transport and metabolism	P	15	4	7	0	0	0	0	0	0	0
Secondary metabolites biosynthesis, transport, and catabolism	Q	5	2	0	0	1	0	0	0	0	0
		POORLY CHARACTERIZED									
General function prediction only	R	0	0	0	0	0	0	0	0	0	0
Function unknown	S	108	19	18	1	0	0	8	0	0	1

* FDR <0.01 $\log_2$ fold change

Differentially expressed genes in individual comparison 24 hr vs 48 hr.

^ Differentially expressed genes in ST comparison 7453 vs 8116 at 24 hrs and 7453 vs 8116 at 48 hr.

Appendix B

Chapter 4 - Characterisation of *Listeria monocytogenes* food-associated isolates to assess environmental fitness and virulence potential.

Supplementary Table B1. Isolate information.

Isolate	MLST ST	Serotype	Year isolated	Description	Lineage	CC	Genebank Accession #
7425	121	1/2a	2011	Environmental - Meat	II	CC121	
7452	155	1/2a, 3a	2009	Dairy - Milk	II	CC155	NAUM00000000
7453	101	1/2a, 3a	2009	Dairy - Cheese	II	CC101	NAUH00000000
7456	155	1/2a, 3a	2010	Dairy - Milk	II	CC155	NAUL00000000
7461	3	1/2b, 3b	2010	Dairy - Milk	I	CC3	
7475	121	1/2a, 3a	2011	Dairy - Cheese	II	CC121	NAUF00000000
7479	1	4b, 4d, 4e	2012	Dairy - Cheese	I	CC1	
7484	101	1/2a, 3a	2012	Dairy - Cheese	II	CC101	NAUG00000000
7488	204	1/2a, 3a	2012	Dairy - Cheese	II	CC204	LXQX00000000
7495	121	1/2a, 3a	2012	Dairy - Cheese	II	CC121	NAUE00000000
7514	3	1/2b, 3b	1998	Vegetable	I	CC3	
7523	1	4b, 4d, 4e	1988	Meat - Beef	I	CC1	NAVA00000000
7530	2	4b, 4d, 4e	1998	Dairy - Ice cream	I	CC2	
7533	155	1/2a	2007	Environmental - Meat	II	CC155	
7535	9	1/2c	2007	Environmental - Meat	II	CC9	LJPE01000000
7536	1	4b, 4d, 4e	2009	Environmental - Dairy	I	CC1	LJPF01000000
7538	2	4b, 4d, 4e	2009	Environmental - Dairy	I	CC2	
7540	7	1/2a, 3a	2011	Mixed Food	II	CC7	
7544	204	1/2a, 3a	2006	Environmental - Dairy	II	CC204	LXQZ00000000
7545	2	4b, 4d, 4e	2008	Mixed Food	I	CC2	
7546	12	1/2a, 3a	2009	Environmental - Dairy	II	CC7	
7547	3	1/2b, 3b	2009	Dairy - Cream	I	CC3	
7550	1	4b, 4d, 4e	2013	Dairy - Cheese	I	CC1	
7553	3	1/2b	2007	Environmental - Meat	I	CC3	
7583	3	1/2b, 3b	2007	Environmental - Dairy	I	CC3	NAUZ00000000
7919	204	1/2a	2015	Meat - Boots	II	CC204	LXRA00000000
7920	155	1/2a	2015	Meat - Boots	II	CC155	
7921	155	1/2a	2015	Meat - Boots	II	CC155	

7922	8	1/2a	2015	Meat - RTE	II	CC8	
7929	204	1/2a, 3a	2015	Meat - Raw Ingredient	II	CC204	LXRB00000000
7943	321	1/2a, 3a	2015	Meat - RTE	II	CC321	
7945	204	1/2a or 3a	2015	Meat - RTE	II	CC204	LXRC00000000
7987	121	1/2a	2016	Meat	II	CC121	
8112	8	1/2a,3a	2010	Meat	II	CC8	SRR6457844
8113	121	1/2a,3a	2009	Environment - Equipment	II	CC121	SRR6457840
8114	101	1/2a,3a	2009	Vegetable	II	CC101	SRR6457839
8115	101	1/2a,3a	2009	Vegetable	II	CC101	SRR6457842
8116	101	1/2a,3a	2009	Vegetable	II	CC101	SRR6457841
8117	9	1/2c,3c	2010	Meat	II	CC9	SRR6457815
8118	9	1/2c,3c	2010	Meat	II	CC9	SRR6457814
8119	9	1/2c,3c	2010	Meat	II	CC9	SRR6457813
8120	8	1/2a,3a	2010	Meat	II	CC8	SRR6457836
8121	8	1/2a,3a	2011	Meat	II	CC8	SRR6457831
8122	1	4b,4d,4e	2011	Meat	I	CC1	SRR6457825
8123	9	1/2c,3c	2011	Vegetable	II	CC9	SRR6457828
8124	8	1/2a,3a	2011	Environment - Floor	II	CC8	SRR6457823
8125	7	1/2a,3a	2011	Meat	II	CC7	SRR6457820
8126	321	1/2a,3a	2011	Environment - Floor	II	CC321	SRR6457864
8127	7	1/2a,3a	2011	Meat	II	CC7	SRR6457859
8128	7	1/2a,3a	2011	Meat	II	CC7	SRR6457868
8129	9	4b,4d,4e	2011	Environment - Wall	II	CC9	SRR6457857
8130	7	1/2a,3a	2012	Environment - Floor	II	CC7	SRR6457877

Supplementary Table B2. Genomic composition.

Isolate	MLST (ST)	Lineage	CC	GC (%)	Number of contigs	Genome size (bp)	Number of CDS
7479	1	I	CC1	38	123	2,864,913	2898
7523	1	I	CC1	38	154	2,918,384	2992
7536	1	I	CC1	38.1	142	2,859,796	2896
7550	1	I	CC1	37.9	49	2,915,699	2948
8122	1	I	CC1	37.7	15	3,032,276	3043
7453	101	II	CC101	38	94	2,949,015	3015
7484	101	II	CC101	38	178	2,933,416	3041
8114	101	II	CC101	37.8	26	3,060,327	3139
8115	101	II	CC101	37.8	25	3,081,702	3165
8116	101	II	CC101	37.8	23	3,060,070	3140
7425	121	II	CC121	37.8	32	3,063,112	3086
7475	121	II	CC121	38	201	2,964,031	3021
7495	121	II	CC121	37.9	126	3,009,752	3086
7987	121	II	CC121	37.8	27	3,053,465	3082
8113	121	II	CC121	37.8	32	3,029,864	3050
7452	155	II	CC155	38	149	2,838,647	2884
7456	155	II	CC155	38	418	2,842,292	2990
7533	155	II	CC155	38	115	2,927,002	2957
7920	155	II	CC155	37.9	15	2,991,163	2979
7921	155	II	CC155	37.9	87	3,048,424	3090
7530	2	I	CC2	37.9	57	2,963,116	2976
7538	2	I	CC2	38	139	2,964,772	3042
7545	2	I	CC2	37.9	62	2,893,120	3021
7488	204	II	CC204	38	200	2,915,997	2980
7544	204	II	CC204	37.9	103	2,909,789	2931
7919	204	II	CC204	37.8	23	2,970,483	2990
7929	204	II	CC204	37.9	62	2,997,489	2993
7945	204	II	CC204	38	32	2,952,310	2966
7461	3	I	CC3	38	99	2,839,705	2868
7514	3	I	CC3	37.9	57	3,014,840	3008
7547	3	I	CC3	38	230	2,939,501	3040
7553	3	I	CC3	38.1	112	2,911,190	2889
7583	3	I	CC3	37.9	56	3,067,839	3082
7943	321	II	CC321	37.8	21	2,952,746	2930
8126	321	II	CC321	37.8	18	3,061,817	3090
7540	7	II	CC7	38.1	144	2,771,113	2783

7546	12	II	CC7	38	153	2,798,393	2849
8125	7	II	CC7	37.9	19	2,926,886	2950
8127	7	II	CC7	37.9	18	2,928,404	2950
8128	7	II	CC7	37.9	18	2,929,391	2947
8130	7	II	CC7	37.9	16	2,960,135	2996
7922	8	II	CC8	37.8	24	3,018,913	3046
8112	8	II	CC8	37.8	20	3,016,802	3049
8120	8	II	CC8	37.8	15	2,613,136	2668
8121	8	II	CC8	37.9	20	2,930,826	2956
8124	8	II	CC8	37.8	20	3,063,629	3127
7535	9	II	CC9	38	111	2,953,641	3006
8117	9	II	CC9	37.8	21	3,012,954	3054
8118	9	II	CC9	37.8	19	3,011,871	3046
8119	9	II	CC9	37.8	19	2,938,491	3014
8123	9	II	CC9	37.9	27	3,063,391	3104
8129	9	II	CC9	37.8	45	3,082,061	3158

Supplementary Table B3. FPE stressor determinants.

		Class I heat shock						Class III heat shock			Cold shock				Osmotic stress		
Isolate	MLST (CC)*	*hrcA*	*grpE*	*dnaK*	*dnaJ*	*groES*	*groEL*	*ctsR*	*clpCP*	*clpE*	*cspA*	*cpsB*	*cspD*	*cpsL*	*gbuABC*	*opuCABCD*	*betL*
7479	1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7523	1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7536	1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7550	1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8122	1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7530	2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7538	2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7545	2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7461	3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7514	3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7547	3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7553	3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7583	3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7540	7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8125	7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8127	7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8128	7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8130	7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

7922	8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8112	8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8120	8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8121	8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8124	8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7535	9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8117	9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8118	9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8119	9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8123	9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8129	9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7546	12 (7)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7453	101	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7484	101	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8114	101	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8115	101	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8116	101	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7425	121	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7475	121	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7495	121	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7987	121	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

8113	121	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7452	155	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7456	155	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7533	155	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7920	155	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7921	155	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7488	204	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7544	204	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7919	204	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7929	204	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7945	204	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7943	321	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8126	321	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

* Clonal complex provided if it is different to MLST ST.

Supplementary Table B3 cont. FPE stressor determinants.

		Acid stress				Bacteriocin/nisin resistance							Oxidative stress					HHP
Isolate	MLST (CC)	*gadB*	*gadC*	*virAB*	*virR*	*LisRK*	*LiaRS*	*lmo2229 (PBP)*	*dltAB*	*fri*	*kat*	*sod*	*gltB*	*gltC*	*sigB*	*perR*	*recA*	*clpB*
7479	1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7523	1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7536	1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7550	1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8122	1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7530	2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7538	2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7545	2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7461	3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7514	3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7547	3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7553	3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7583	3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7540	7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8125	7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8127	7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8128	7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8130	7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

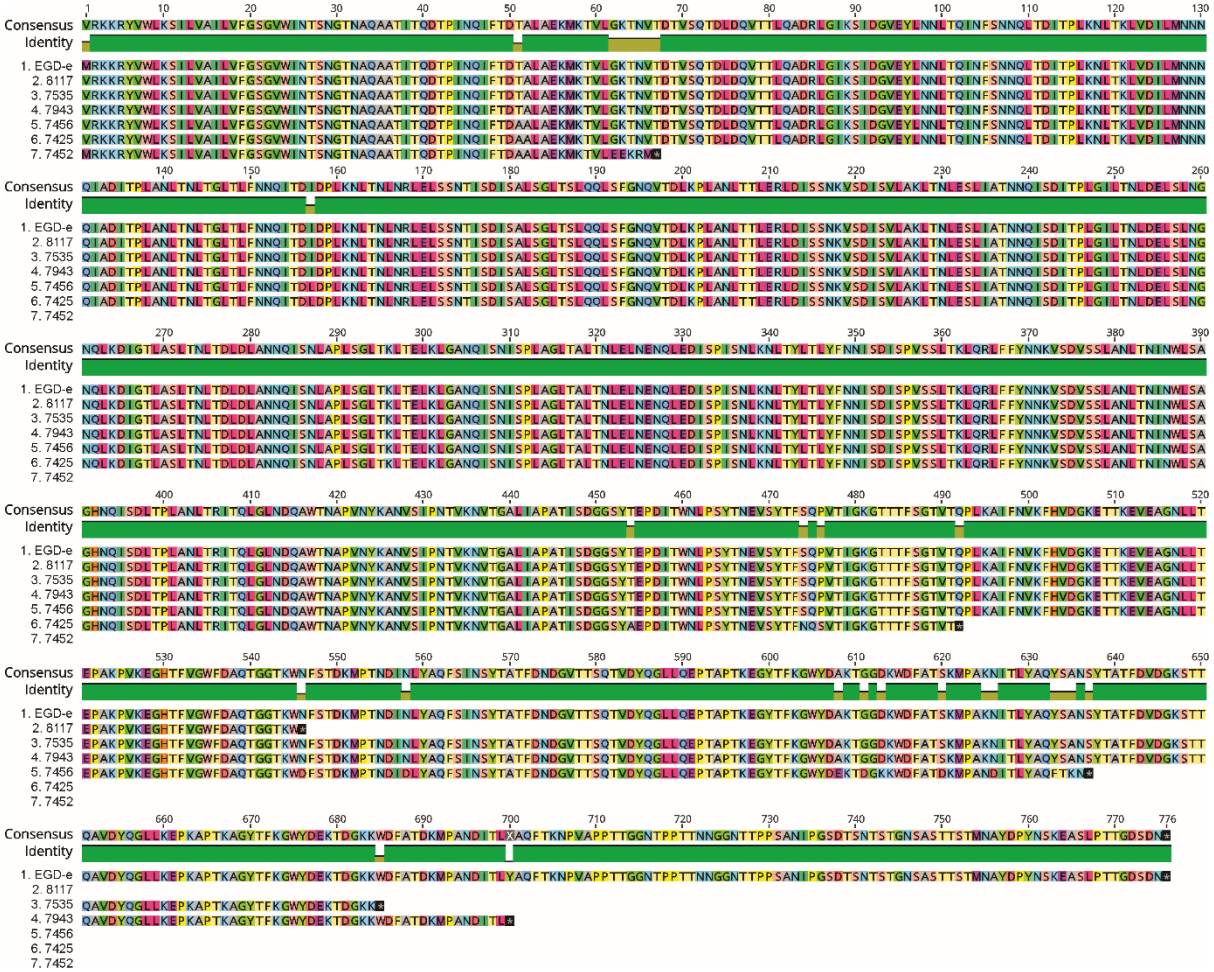
7922	8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8112	8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8120	8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8121	8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8124	8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7535	9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8117	9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8118	9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8119	9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8123	9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8129	9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7546	12 (7)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7453	101	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7484	101	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8114	101	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8115	101	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8116	101	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7425	121	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7475	121	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7495	121	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7987	121	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

8113	121	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7452	155	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7456	155	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7533	155	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7920	155	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7921	155	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7488	204	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7544	204	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7919	204	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7929	204	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7945	204	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7943	321	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8126	321	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

* Clonal complex provided if it is different to MLST ST.

	CadA1	CadA2	CadA7	CadA3	CadA6	CadA4	CadA5
CadA1		67.74%	69.52%	69.89%	64.34%	34.58%	34.48%
CadA2	67.74%		75.74%	73.51%	63.19%	35.29%	35.18%
CadA7	69.52%	75.74%		74.82%	63.89%	34.59%	34.58%
CadA3	69.89%	73.51%	74.82%		63.85%	34.68%	33.89%
CadA6	64.34%	63.19%	63.89%	63.85%		35.61%	35.41%
CadA4	34.58%	35.29%	34.59%	34.68%	35.61%		89.71%
CadA5	34.48%	35.18%	34.58%	33.89%	35.41%	89.71%	

Supplementary Figure B1. Pairwise amino acid percentage identity comparison of the cadmium resistance determinants *cadA1-A7*.



Supplementary Figure B2. Protein alignment of isolates representing *inlA* mutations identified in this study. The first line is the full length *inlA* identified from EGD-e and used as the reference; line 2 represents mutation type 12 identified in three isolates; line 3 represents mutation type 11 identified in two isolates; line 4 is mutation type 3 also present in two isolates; line 5 is a 70 AA deletion present in one isolate only; line 6 is mutation type 6 present in five isolates; and line 7 is a novel mutation identified in one isolate only and referred to as mutation type 22.

Supplementary Table B4. Antibiotic resistance determinants.

		Fluoroquinolone 2nd G						Aminoglycosides				Pencillin					Amoxicillin		Trimethoprim		Sulfonamide	Vancomycin	
Isolate	MLST (CC)	*fepA*	*fepR*	*gyrA*	*gyrB*	*parC*	*parE*	*spc*	*strA*	*aadE*	*aadA*	*lmo0540*	*lmo1892* (*PBP2A*)	*lmo2039* (*PBP2B*)	*PBP1A*	*mecC*	*blaTEM1*	*blaTEM116*	*dfrD*	*drfG*	*sulI*	*vanA*	*vanB*
7479	1	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-
7523	1	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-
7536	1	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-
7550	1	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-
8122	1	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-
7530	2	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-
7538	2	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-
7545	2	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-
7461	3	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-
7514	3	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-
7547	3	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-
7553	3	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-
7583	3	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-
7540	7	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-
8125	7	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-
8127	7	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-
8128	7	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-
8130	7	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-
7922	8	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-
8112	8	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-
8120	8	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-

8121	8	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-
8124	8	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-
7535	9	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-
8117	9	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-
8118	9	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-
8119	9	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-
8123	9	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-
8129	9	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-
7546	12 (7)	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-
7453	101	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-
7484	101	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-
8114	101	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-
8115	101	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-
8116	101	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-
7425	121	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-
7475	121	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-
7495	121	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-
7987	121	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-
8113	121	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-
7452	155	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-
7456	155	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-
7533	155	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-
7920	155	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-
7921	155	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-
7488	204	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-

7544	204	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-
7919	204	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-
7929	204	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-
7945	204	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-
7943	321	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-
8126	321	+	+	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	+	-	-

* Clonal complex provided if it is different to MLST ST.
+; present, blank; absent.

Supplementary Table B4. Antibiotic resistance determinants cont.

		Tetracycline						Erythomycin									
Isolate	MLST (CC)	*tetL*	*tetM*	*tetS*	*tetA*	*tetK*	MFS efflux pump	*ermA*	*ermB*	*ermC*	*fosX*[1]	*lmrB*[2]	*oatA*[3]	*rplF*[4]	*mprF*[5]	*norB*[6]	*ampC/pbpX*[7]
7479	1	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+
7523	1	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+
7536	1	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+
7550	1	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+
8122	1	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+
7530	2	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+
7538	2	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+
7545	2	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+
7461	3	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+
7514	3	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+
7547	3	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+
7553	3	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+
7583	3	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+
7540	7	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+
8125	7	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+
8127	7	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+
8128	7	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+
8130	7	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+
7922	8	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+
8112	8	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+

8120	8	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+
8121	8	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+
8124	8	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+
7535	9	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+
8117	9	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+
8118	9	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+
8119	9	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+
8123	9	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+
8129	9	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+
7546	12 (7)	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+
7453	101	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+
7484	101	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+
8114	101	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+
8115	101	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+
8116	101	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+
7425	121	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+
7475	121	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+
7495	121	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+
7987	121	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+
8113	121	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+
7452	155	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+
7456	155	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+
7533	155	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+
7920	155	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+
7921	155	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+

7488	204	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+
7544	204	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+
7919	204	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+
7929	204	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+
7945	204	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+
7943	321	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+
8126	321	-	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+

* Clonal complex provided if it is different to MLST ST.
1 Fosfomycin
2 Lincosamides
3 Aceyltransferase
4 Fusidine
5 Antibiotic peptide – integral membrane/defensin resistance
6 Quinolone
7 Cephalosporins
+; present, blank; absent.

Appendix C

Published versions of chapters 2-5

The following has been removed for copyright or proprietary reasons.

Gray, J A., Chandry, P. S., Bowman, J. P., Fox, E. M. 2021. High-throughput screening of biofilm formation of Listeria monocytogenes on stainless steel coupons using a 96-well plate format, in: Fox, E. M., Bierne, H., Stessl, B. (eds) Listeria monocytogenes, Methods in molecular biology, vol 2220. © Springer Science+Business Media, LLC, part of Springer Nature 2021 New York, NY. https://doi.org/10.1007/978-1-0716-0982-8_9.

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Colonisation dynamics of *Listeria monocytogenes* strains isolated from food production environments

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Listeria monocytogenes **is a ubiquitous bacterium capable of colonising and persisting within food production environments (FPEs) for many years, even decades. This ability to colonise, survive and persist within the FPEs can result in food product cross-contamination, including vulnerable products such as ready to eat food items. Various environmental and genetic elements are purported to be involved, with the ability to form biofilms being an important factor. In this study we examined various mechanisms which can influence colonisation in FPEs. The ability of isolates (n = 52) to attach and grow in biofilm was assessed, distinguishing slower biofilm formers from isolates forming biofilm more rapidly. These isolates were further assessed to determine if growth rate, exopolymeric substance production and/or the *agr* signalling propeptide influenced these dynamics and could promote persistence in conditions reflective of FPE. Despite no strong association with the above factors to a rapid colonisation phenotype, the global transcriptome suggested transport, energy production and metabolism genes were widely upregulated during the initial colonisation stages under nutrient limited conditions. However, the upregulation of the metabolism systems varied between isolates supporting the idea that *L. monocytogenes* ability to colonise the FPEs is strain-specific.**

Listeria monocytogenes is a Gram-positive foodborne pathogen which can cause the life-threatening disease listeriosis, particularly in at-risk populations. While listeriosis is an uncommon food borne illness, in the at-risk population group covering immunocompromised, elderly, pregnant women and neonates, the mortality rate can reach as high as 30%[1–3]. As the food supply chain has become progressively more global, increased reports of multistate and international food recalls and outbreaks are occurring, therefore the need to understand *L. monocytogenes* ability to colonise and persist in food processing environments (FPEs) is paramount[4]. Traditionally the presence of *L. monocytogenes* in food products has been associated with foods like ready to eat meats, seafood products, unpasteurised milk and dairy products, however new food items like melons, various fresh, pre-cut and frozen fruit and vegetables, leafy greens, sandwiches and wraps are now being linked to *L. monocytogenes*[4]. The ubiquitous nature of this foodborne bacterium makes it difficult to control and manage, and due to this can be repeatedly introduced into FPEs[5] and therefore efforts should be targeted towards this environment. It is not uncommon for reports of persistent strains to arise with studies describing the isolation of some strains over numerous years[6–9]. The presence of persistent strains in the FPE can act as a repetitive source of contamination and imply the cleaning and sanitation program is not always effective in their control. Persistence within the FPE is suspected to be linked to a variety of factors including resistance and tolerance to disinfectants, acid and heat applications, favourable niches due to poor facility design and condition, along with the ability to attach to a variety of surfaces and the formation of biofilms[10–13].

Biofilms consist of microbial cells, generally multi-species, attached to each other or a surface, and surrounded by an extracellular polymeric substance which provides increased fitness to all cells within the biofilm[14–16].

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Time point (h)	log_{10} CFU/cm^2	Variation in cell numbers (log_{10} CFU/cm^2)
24	1.20–4.16	2.95
48	2.61–5.39	2.78
72	4.51–5.83	1.32
96	4.40–5.82	1.41

Table 1. Mean biofilm density (log_{10} CFU/cm^2) range of all 52 *L. monocytogenes* biofilm isolates at sampled timepoints.

Biofilms provide increased protection from cleaning agents, disinfectants and desiccation, enhances the transfer of nutrients and removal of toxic metabolites, and increases the opportunity to acquire new genetic traits[14–16]. The process of attachment and biofilm formation in *L. monocytogenes* has been reported[17–19], however there is less consensus on what specific genes are responsible for *L. monocytogenes* ability to colonise and survive in FPEs, and it is likely a synergy of multiple mechanisms are involved. The aims of this study were to develop a simplified model system to reflect the FPE in terms of contact surface, temperature and limited nutrient availability, key conditions in FPEs. This simplified model system was then used to determine: (i) if there were any differences in the early stages of biofilm formation between *L. monocytogenes* strains isolated from various food and environmental sources for multi-locus sequence types (MLST) commonly associated with FPEs; (ii) if there are genes or phenotypes associated with the biofilm phenotype; (iii) if there are differences in expression levels of the signalling associated *agrD* gene, known to be involved in adherence, between fast and slow biofilm formers and; (iv) if there are differences in transcription levels of genes between two MLST STs both present in the slow and fast biofilm groups.

Results

Isolate characteristics. The strains included in this study were isolated from food or related environments, including meat, dairy, vegetable, and mixed sources, across a span of 18 years from 1998 to 2016 (Supplementary Table 1). The draft genome sizes of the 52 *L. monocytogenes* isolates ranged from 2.61 to 3.08 Mb, with the GC percentage between 37.7 and 38.1%.

Biofilm formation on SS coupons. Biofilm formation was assessed on SS coupons at 24, 48, 72 and 96 h. Isolates were examined based on mean biofilm cell density (log_{10} CFU/cm^2) with a broad distribution observed at each timepoint (Table 1) indicating all isolates were able to form biofilms. Greatest differences in mean biofilm density between strains was observed at 24 and 48 h (Supplementary Fig. 1). A fast biofilm forming group with cell densities of 3.5–4.2 log_{10} CFU/cm^2 formed after 24 h was well separated from a slow biofilm forming isolates which only reached 1.2–1.8 log_{10} CFU/cm^2 after 24 h (Fig. 1). This separation was less evident by 72 and 96 h with the mean cell densities being within 1.5 log_{10} when comparing isolates. The five isolates with the highest biofilm cell densities at 24 h were deemed to be the fast biofilm forming group (appended with ^{F} for clarity) consisted of 7921^F, 7453^F, 7425^F, 7545^F and 7546^F. The five isolates with the lowest biofilm cell densities at 24 h were considered slow biofilm formers (appended with ^{S}) and included 7488^S, 8116^S, 7536^S, 7514^S and 7538^S; together, these fast and slow biofilm groupings make up the B10 isolates. At 24 h, three of the five isolates in the slow biofilm forming group were from Lineage I and four of the five isolates from the fast biofilm forming group were from Lineage II. Two of the isolates from the fast biofilm forming group were from MLST ST155. MLST ST101 and ST2 had an isolate in both groups whereas all the other isolates in the two groups were from different STs.

EPS production. The ability to produce exopolymeric substances was assessed in the B10 group to investigate if these features influence the ability of isolates to attach and form biofilms faster. In this study, isolates which showed a pink phenotype at 14 °C regardless of the growth media were 7425^F, 7453^F, 7488^S, 7514^S, 7536^S and 7538^S illustrating an intermediate ability to bind Congo red and thus produce some form of EPS (Supplementary Fig. 2). A translucent phenotype was displayed by isolates 7456^F, 7545^F, 7921^F and 8116^S indicating they were unable to bind the Congo red dye and therefore did not produce EPS. At 37 °C most isolates displayed the same phenotype as they did at 14 °C although some changed phenotype, isolates 7453^F and 7488^S which became translucent, and 7456^F which produced a pink phenotype. Isolates 7536^S and 7538^S changed from the pink phenotype to translucent at 37 °C when grown in MHB. The above phenotypes were not associated with a slow or fast biofilm formation group.

Growth rate and doubling time of B10. The growth rate of the B10 isolates at 14 °C in dBHI and at 37 °C in BHI was determined (Fig. 2). At 14 °C the isolates growth rate ranged from 0.00060 and 0.00093 min^{-1}. The slowest growth rate was associated with isolate 7545^F with the fastest growth rate belonging to isolate 7488^S. The doubling time was also measured with a broad range of times observed (12.4–19.9 h). At 37 °C the growth rate and doubling times ranged from 0.01315 to 0.01468 min^{-1} and 43.55 to 48.61 min, respectively, reflective of typical *L. monocytogenes* growth under optimal conditions. Importantly, growth rate and doubling times were not correlated to biofilm forming ability at either temperature.

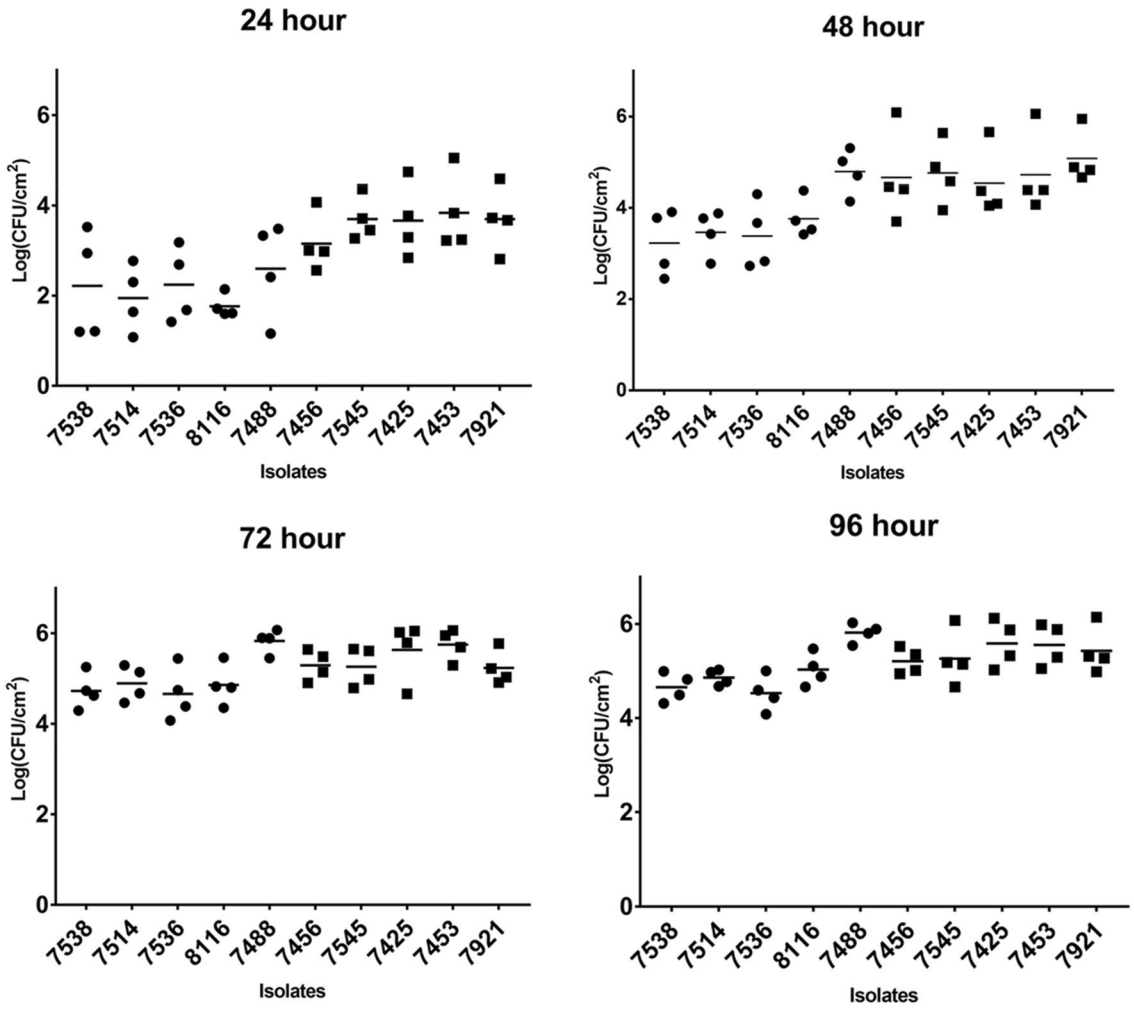

Figure 1. Comparison of the B10 isolates identified as displaying faster or slower biofilm formation over 96 h. Biofilm density (log_{10} CFU/cm^2) was determined every 24 h by standard plate count. Data points represent the average of 4 biological experiment replicates. Dots, slow isolates; squares, fast isolates.

Genome wide association study. A microbial genome wide association study was performed across the 52 isolates utilising the biofilm phenotypic data to assess if there were any genetic differences associated with biofilm formation and attachment ability. No significant single nucleotide polymorphisms (SNPs) were associated with a faster or slower biofilm formation phenotype were identified amongst the 52 isolates; similarly, no genes showed statistically significant phenotypic association. Phylogenetic association was determined by treeWAS based upon 28,414 core SNPs resulting in isolates grouping by clonal complex (Supplementary Fig. 3).

***agrD* gene expression.** The expression levels of the propeptide *agrD* was assessed using qRTi-PCR in the B10 isolates on coupons and in SM at 24 and 48 h. The Wilcoxon rank sum test indicated there was no statistically significant difference in *agrD* expression when comparing the fast and slow isolates against the independent growth conditions and timepoints. When the *agrD* expression is assessed by either paired condition or timepoint some differences are determined (Fig. 3). Notably, *agrD* expression was upregulated in the early stages of attachment and biofilm development, relative to other conditions tested.

Transcriptional analysis. *Differentially expressed genes (DEGs) under food production environment biofilm formation conditions.* The global transcriptomic changes in biofilm formation at 24 h and 48 h in dBHI was assessed against four individual isolates with two isolates from both the slow and fast biofilm formation groups, respectively. The isolates chosen represented ST101 and ST2 with a fast and slow isolate in each ST. The number of reads ranged from 21,954,948 to 65,818,623 and were mapped to each isolate's individual genome. A total of 494 differentially expressed genes (DEGs) were identified using a false discovery rate (FDR) of < 0.01 and log fold change (logFC) of ≥ 2 across all comparisons. Isolate 7538^S and ST2 at both timepoints had no DEGs which met the FDR and log_2FC cut-off. At 24 h isolates 7453^F, 7545^F and 8116^S had 286, 76 and 7 DEGs respectively resulting in a total of 369 up regulated DEGs. At 48 h isolates 7453^F and 7545^F had 85 and 23 DEGs respectively

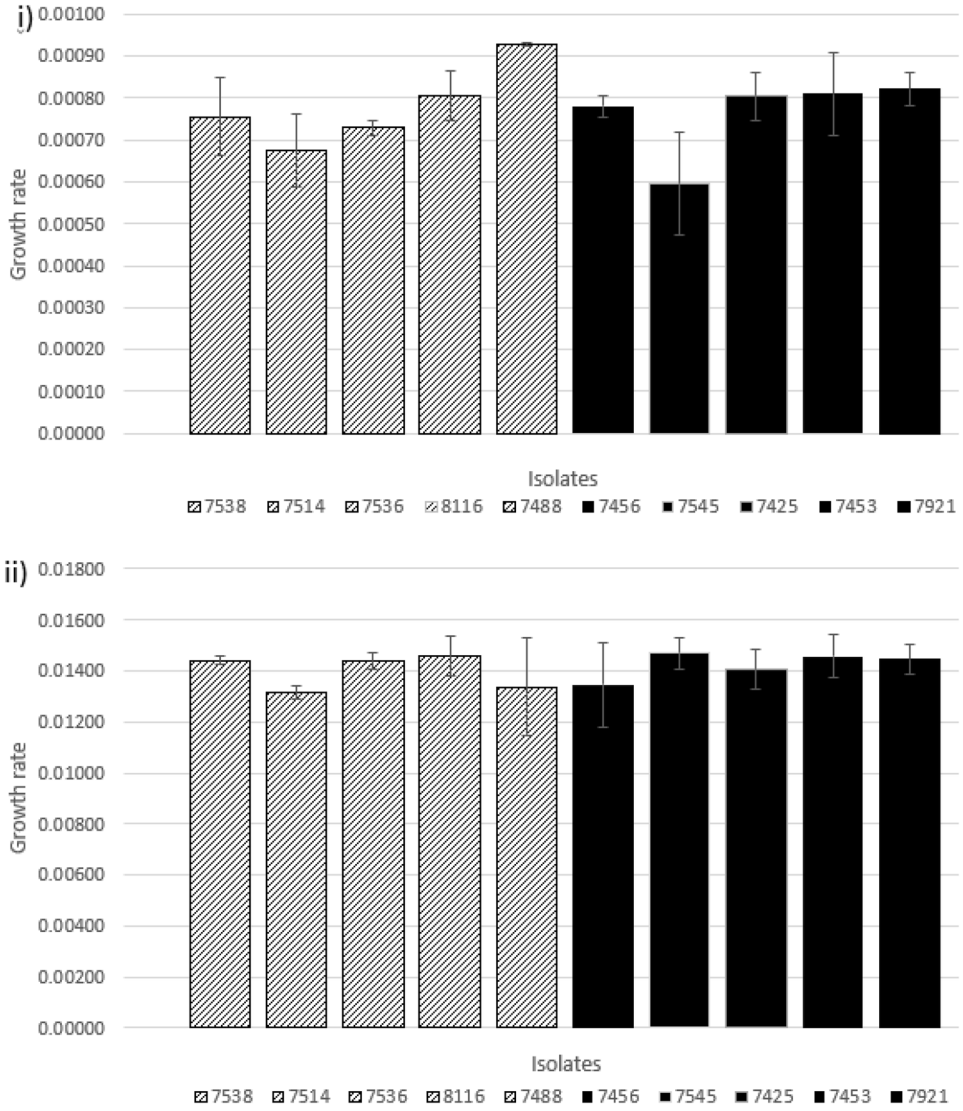

Figure 2. Mean specific growth rate of the B10 isolates and standard deviation at: (**i**) 14 °C in dBHI (three biological replicates) and; (**ii**) 37 °C in full BHI (two biological and seven technical replicates). Patterned bars—slow isolates; solid black bars—fast isolates.

totalling 108 DEGs. Between ST101 there was 11 up regulated and 6 down regulated DEGs at 24 h and 48 h respectively. The DEGs were annotated in Eggnog.

Functional annotation of transcriptome. The clusters of orthologous groups (COGs) were used to identify the functional categories of the DEGs. The DEGs were allocated to 20 of the COG categories (Supplementary Table S2) with 19 DEGs assigned to multiple (>1) COG categories and were therefore treated as belonging to both. Approximately a third (30%) of the DEGs were allocated to the 'S' COG categories 'Function unknown'. Amongst the 24 h up regulated DEGs with functional assignments the next three prevalent COG categories are 'G: Carbohydrate transport and metabolism', 'J: Translation, ribosomal structure and biogenesis', and 'M: Cell wall/membrane/envelope biogenesis.' The top three amongst 48 h up regulated DEGs also includes categories 'G' and 'J' along with 'K: Transcription'.

Pathways identified. The STRING database was used to identify overexpressed pathways and the molecular mode of action present within the DEGs of isolates 7453^{F}, 7545^{F} and ST101 at 24 and 48 h. (Fig. 4 and Table 2). In isolate 7453^{F}, the phosphotransferase system (PTS) (FC range 3.48–6.10) and starch and sucrose metabolism pathways (FC range 3.66–6.10) were overexpressed at 24 h along with cobalamin biosynthesis (FC range 3.35–5.05). The pathways for amino sugar and nucleotide sugar metabolism (FC range 2.99–3.89) were overexpressed at 48 h. The overexpressed pathway identified in isolate 7545^{F} at 24 h included starch and sucrose metabolism (FC range 2.87–3.69). At 48 h the pathway overexpressed was ribosome (FC range 3.36–4.56) associated with

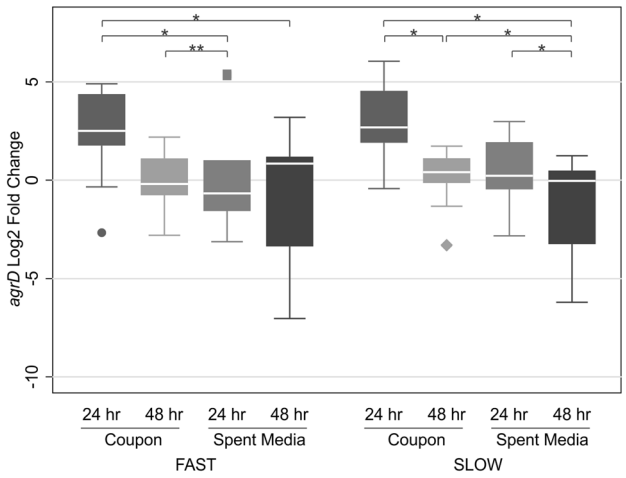

Figure 3. *agrD* expression (log_2 fold change) at 24 and 48 h in coupons and spent media. No statistically significant difference in *agrD* expression between slow and fast isolates. Comparison of paired conditions or timepoints displayed significant difference, specifically C24hr Fast and SM24hr Fast ($Z = 2.073$, $p = 0.0382$), C48hr Slow and SM48hr Slow ($Z = 1.992$, $p = 0.0464$), C24hr Slow and C48hr Slow ($Z = 2.490$, $p = 0.0128$), SM24hr Slow and SM48 Slow ($Z = 2.192$, $p = 0.0284$), C24hr Fast and SM48hr Fast ($Z = 2.341$, $p = 0.0192$), C48hr Fast and SM24hr Fast ($Z = 2.970$, $p = 0.0030$) and C24hr Slow and SM48hr Slow ($Z = 2.521$, $p = 0.0117$). *$p < 0.05$; **$p < 0.01$. *C* coupon, *SM* spent media, *Z* z score, shading refers to the different experimental conditions.

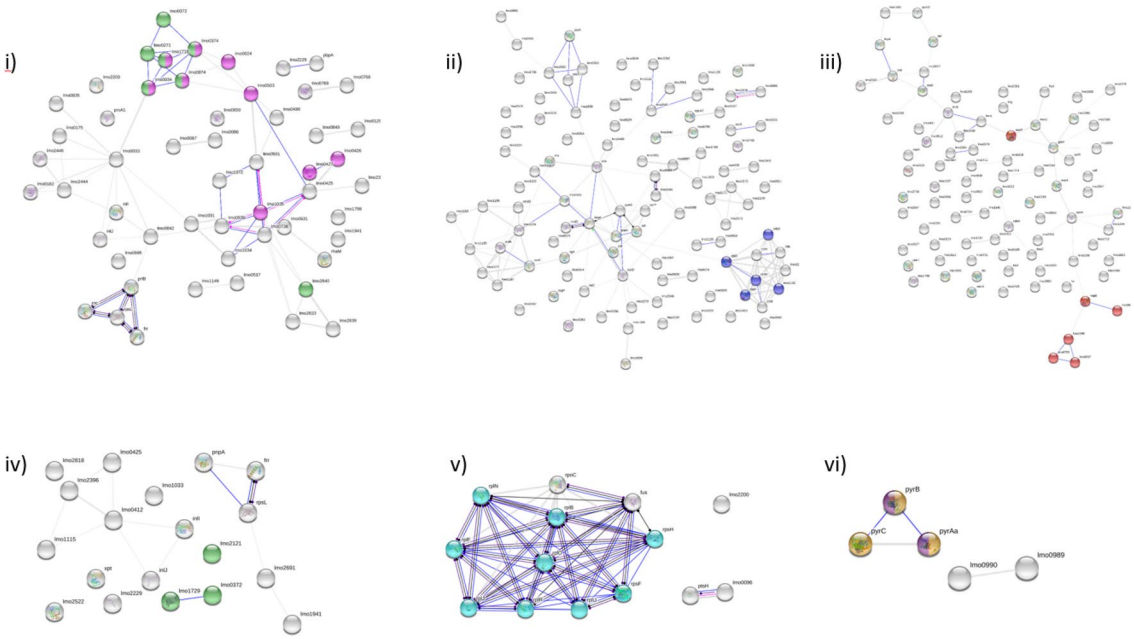

Figure 4. Overexpressed protein pathways in the transcriptome at 24 and 48 h in isolates 7453, 7545 and ST101 48 h. (**i** and **ii**) 7453 24 h; (**iii**) 7453 48 h; (**iv**) 7545 24 h; (**v**) 7545 48 h; (**vi**) ST101 48 h. Coloured nodes relate to overexpressed pathways: pink, phosphotransferase system; light green, starch and sucrose metabolism; dark blue, cobalamin biosynthesis; red, amino sugar and nucleotide sugar metabolism; light blue, ribosome; yellow, pyrimidine metabolism; and purple, alanine, aspartate and glutamate metabolism. Coloured lines connecting nodes relate to action type: blue, binding; black, reaction; purple, catalysis; and pink, post-translation modification. Locus tags and genes names are based upon matches to proteins in the reference genome, *L. monocytogenes* EGD-e.

various RNA proteins and ribosomal domains identified. Pyrimidine metabolism and alanine, aspartate and glutamate metabolism (FC range 4.20–4.37) were pathways overexpressed for the ST101 48 h down regulated DEGs (Table 3). The upregulated DEGs of ST101 at 24 h predominately consisted of prophage genes (FC range 9.61–12.10). In addition, isolate 7453^F at 24 h also contain prophage up regulated DEGs (FC range 3.34–6.20). Most of the molecular action consisted of post translational modification, reaction, binding and catalysis.

Locus tag (EGD-e)	Locus tag (this study)	Fold change	Gene	COG cat	Description	KEGG enzyme	Isolate and TP#
PTS system							
lmo1035	fig|1639.4014.peg.1355	4.22		G	PEP-dependent sugar PTS, EIIA 1		7453 24 h
lmo1719	fig|1639.4014.peg.1473	4.36		G	PTS system cellobiose-specific IIA component	2.7.1.205	
lmo0427	fig|1639.4014.peg.2115	4.77		G	PTS system, Lactose/Cellobiose specific IIB subunit		
lmo0426	fig|1639.4014.peg.2116	4.02		G	PEP-dependent sugar PTS, EIIA 2		
lmo0024	fig|1639.4014.peg.434	4.99		G	PTS system mannose/fructose/sorbose family IID component		
lmo0034	fig|1639.4014.peg.444	5.61		G	PTS system cellobiose-specific IIC component		
lmo0374	fig|1639.4014.peg.610	3.66		G	PTS system cellobiose-specific IIB component	2.7.1.205	
lmo0874	fig|1639.4014.peg.773	6.1		G	PTS system, Lactose/Cellobiose specific IIA subunit; PTS system beta-glucoside-specific IIA component		
lmo0503	fig|1639.4014.peg.936	3.48		G	PTS system galactitol-specific IIA component	2.7.1.200	
Starch and sucrose metabolism							
lmo0271	fig|1639.4014.peg.1428	4.47		G	Glycosyl hydrolase 1 family; 6-phospho-beta-glucosidase	3.2.1.86	7453 24 h
lmo1719	fig|1639.4014.peg.1473	4.36		G	PTS lichenan-specific enzyme IIA component; PTS system beta-glucoside-specific IIA component; PTS system cellobiose-specific IIA component	2.7.1.205	
lmo2840	fig|1639.4014.peg.393	4.82	YcjM	G	Sucrose glucosyltransferase/sucrose phosphorylase (ycjM)	2.4.1.7	
lmo0034	fig|1639.4014.peg.444	5.61		G	PTS system cellobiose-specific IIC component		
lmo0372	fig|1639.4014.peg.608	4.16		G	Glycosyl hydrolase 1 family; 6-phospho-beta-glucosidase	3.2.1.86	
lmo0374	fig|1639.4014.peg.610	3.66		G	PTS system cellobiose-specific IIB component	2.7.1.205	
lmo0874	fig|1639.4014.peg.773	6.1		G	PTS system, Lactose/Cellobiose specific IIA subunit; PTS system beta-glucoside-specific IIA component		
lmo1729	fig|1639.4024.peg.1538	3.27		G	Glycosyl hydrolase 3 family; beta-glucosidase	3.2.1.21	7545 24 h
lmo0372	fig|1639.4024.peg.670	3.69		G	Glycosyl hydrolase 1 family; 6-phospho-beta-glucosidase	3.2.1.86	
lmo2121	fig|1639.4024.peg.985	2.87		G	Trehalose and maltose hydrolases; Maltose phosphorylase	2.4.1.8	
Cobalamin biosynthesis							
lmo1148	fig|1639.4014.peg.281	5.05	cobS	H	Cobalamin synthase	2.7.8.26	7453 24 h
lmo1192	fig|1639.4014.peg.237	3.51	cobD	H	Adenosylcobinamide-phosphate synthase	6.3.1.10	
lmo1194	fig|1639.4014.peg.235	3.95	cbiD	H	Cobalt-precorrin-5B (C1)-methyltransferase	2.1.1.195	
lmo1191	fig|1639.4014.peg.238	3.35	cbiA	H	Cobyrinic acid c-diamide synthetase	6.3.5.11	
lmo1204	fig|1639.4014.peg.225	4.61	cbiM	P	Cobalt ECF transporter substrate-binding protein CbiM		
Prophage related genes							
	fig|1639.4014.peg.1757	3.84		K	BRO family, N-terminal domain; Antirepressor [Bacteriophage A118]		7453 24 h
	fig|1639.4014.peg.1796	3.38		N	Bacterial Ig-like domain 2; Protein gp13 [Bacteriophage A118]		
	fig|1639.4014.peg.2230	3.41		S	Phosphoadenosine phosphosulfate; Co-activator of prophage gene expression IbrA		
	fig|1639.4014.peg.2484	5.39		S	Phage protein		
	fig|1639.4014.peg.1804	4.2		S	Putative short tail fibre [Bacteriophage A118]		
	fig|1639.4014.peg.1780	4.16		S	Protein of unknown function (DUF2481) [Bacteriophage A118]		
	fig|1639.4014.peg.2780	4.01		S	Prophage endopeptidase tail		
	fig|1639.4014.peg.1788	3.89		S	Phage minor capsid protein 2		
	fig|1639.4014.peg.1793	3.87		S	Minor capsid protein		
	fig|1639.4014.peg.1805	3.8		S	Protein gp22 [Bacteriophage A118]		
	fig|1639.4014.peg.2062	3.61		S	Phage tail tape measure protein		
	fig|1639.4014.peg.2783	3.41		S	COG5546 Small integral membrane protein		
	fig|1639.4014.peg.1787	3.34		S	Phage portal protein, SPP1 Gp6-like [Bacteriophage A118]		
	fig|1639.4014.peg.1759	6.2		S	Protein gp44 [Bacteriophage A118]		
	fig|1639.4014.peg.468	5.43		V	Type VII secretion protein EsaA		

Continued

Locus tag (EGD-e)	Locus tag (this study)	Fold change	Gene	COG cat	Description	KEGG enzyme	Isolate and TP#
Amino sugar and nucleotide sugar metabolism							
lmo0957	fig|1639.4014.peg.2911	3.89	nagB	G	Glucosamine-6-phosphate deaminase	3.5.99.6	7453 48 h
lmo0956	fig|1639.4014.peg.2912	3.55	nagA	G	N-acetylglucosamine-6-phosphate deacetylase	3.5.1.25	
lmo0096	fig|1639.4014.peg.505	3.03		G	PTS system mannost-specific transporter subunits IIAB	2.7.1.191	
lmo0097	fig|1639.4014.peg.506	2.99		G	PTS system mannose-specific IIC component		
Lmo0783	fig|1639.4014.peg.1297	4.18	manX	G	PTS system mannose-specific IIAB component	2.7.1.191	
lmo2552	fig|1639.4014.peg.192	3.7	murZ	M	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	2.5.1.7	
Ribosome							
lmo1542	fig|1639.4024.peg.1293	3.82	rplU	J	LSU ribosomal protein L21p		7545 48 h
lmo0250	fig|1639.4024.peg.1620	3.36	rplJ	J	LSU ribosomal protein L10p (P0)		
lmo2629	fig|1639.4024.peg.266	4.02	rplB	J	LSU ribosomal protein L2p (L8e)		
lmo2622	fig|1639.4024.peg.273	3.85	rplN	J	LSU ribosomal protein L14p (L23e)		
lmo2618	fig|1639.4024.peg.277	4.56	rpsH	J	SSU ribosomal protein S8p (S15Ae)		
lmo2617	fig|1639.4024.peg.278	4.01	rplF	J	LSU ribosomal protein L6p (L9e)		
lmo2616	fig|1639.4024.peg.279	3.85	rplR	J	LSU ribosomal protein L18p (L5e)		
lmo2613	fig|1639.4024.peg.282	3.57	rplO	J	LSU ribosomal protein L15p (L27Ae)		
lmo0044	fig|1639.4024.peg.808	4.46	rpsF	J	SSU ribosomal protein S6p		

Table 2. Overexpressed pathways in differentially* expressed genes at 24 and 48 h in *L. monocytogenes* isolates 7453^{F} and 7545^{F}. *FDR < 0.01 $\log_2$ fold change. #Time point.

Locus tag (EGD-e)	Locus tag (this study)	FC	Gene	COG Cat	Gene/protein name	KEGG	Isolate and TP#
Prophage related genes							
	fig|1639.4037.peg.3124	9.61		S	Microvirus J protein; Phage DNA binding protein		ST101 24 h up regulated
	fig|1639.4037.peg.3125	10.88		S	Bacteriophage scaffolding protein D		
	fig|1639.4037.peg.3120	10.95		S	Bacteriophage replication gene A protein (GPA)		
	fig|1639.4037.peg.3126	10.99		S	Phage protein C; Phage single stranded DNA synthesis		
	fig|1639.4037.peg.3123	11.06		S	Capsid protein (F protein); Phage major capsid protein		
	fig|1639.4037.peg.3121	11.21		S	Microvirus H protein (pilot protein); Phage minor capsid protein		
	fig|1639.4037.peg.3122	11.83		S	Major spike protein (G protein)		
	fig|1639.4037.peg.3127	12.1		S	Bacteriophage replication gene A protein (GPA)		
Pyrimidine metabolism and Alanine, aspartate and glutamate metabolism							
lmo1838	fig|1639.4037.peg.1939	−4.37	pyrB	F	Aspartate carbamoyltransferase	2.1.3.2	ST101 48 h down regulated
lmo1837	fig|1639.4037.peg.1938	−4.32	pyrC	F	Dihydroorotase	3.5.2.3	
lmo1036	fig|1639.4037.peg.1937	−4.2	pyrAa	F	Carbamoyl-phosphate synthase small chain	6.3.5.5	

Table 3. ST101 pathways overexpressed in differentially* expressed genes at 24 and 48 h in *L. monocytogenes*. *FDR < 0.01 log2 fold change. #Time point.

	7453^{F}		7538^{S}		7545^{F}	
	24 h	48 h	24 h	48 h	24 h	48 h
ctsR				3.61		
degU				3.22		
fur		4.16				
lexA		2.82				
mogR					1.67	
recA		2.56		3.42		2.32
sigB				3.81		

Table 4. Fold change of regulator genes differentially expressed at FDR < 0.05.

Differential expression of select regulator genes. Seven of the regulatory genes selected for their association with stress response were significantly differentially expressed (DE) across three of the isolates (Table 4). Three regulatory genes, *fur*, *lexA* and *recA* were DE in isolate 7453 at 48 h with the logFC range between 2.56 and 4.16. Isolate 7538 displayed DE of four genes, *ctsR*, *degU*, *recA* and *sigB* at 48 h with the logFC ranging from 3.22 to 3.81. The *mogR* gene (logFC 1.67) and *recA* (logFC 2.32) gene was DE in isolate 7545 at 24 h and 48 h respectively. All other time points and isolates were negative for significant differential expression of the selected regulatory genes. Interestingly, *recA* was the only regulatory gene which was DE across three different isolates all at the 48 h timepoint and a logFC range from 2.32 to 3.42.

Discussion

Listeria monocytogenes' ability to colonise FPE is a concern for the health of the at-risk population and the processing facilities' economic viability and reputation. A deeper knowledge of *L. monocytogenes*' ability to colonise and survive in FPE is required. The ability to replicate conditions representative of the FPE will assist in improving our understanding of these dynamics, however there are multiple complex elements involved including the type of contact surfaces (food and non-food) present, temperature, time, nutrients and ability to form biofilms. The availability and type of nutrients varies depending on the type of food or food products processed. While it is difficult to replicate the exact nutritional content available in the FPE, it is known to alternate between high and low nutritional stages during cycles of production. In this study, we assessed colonization behaviour of *L. monocytogenes*, incorporating these factors to reflect those of the FPE.

Initially there was some debate in the literature on *L. monocytogenes* ability to form biofilms, however there is growing evidence to suggest biofilm formation is a key component of the survival and persistence of some strains[16,20–22]. Early studies have tried to associate biofilm formation to a lineage or serotype with varying results[23–25]. In this study, biofilm formation was observed to be strain-specific as there was no consistency in the fast or slow biofilm groups linking a given phenotype to a specific genotype, as discussed below, and initially suggested in earlier *L. monocytogenes* biofilm studies[25–28]. Although two isolates belonging to MLST ST155 were present in the faster biofilm forming group, ST101 and ST2 had an isolate in both faster and slower groups, indicating no clear phenotype association with genetic sub-lineage. As attachment and biofilm formation appears to be environment and strain-specific we sought to determine what additional components may be of influence.

In FPE, access to nutrients can be transient therefore *L. monocytogenes* cells need to be able to adapt to the environmental conditions available. Biofilm formation studies have assessed the impact of nutrient deprivation, such as the study by Kadam et al.[25] reporting enhanced biofilm formation and attachment was positively influenced in nutrient poor media. Cherifi et al.[29] assessed BHI and a diluted BHI media with similar results. The results from this study correlate with *L. monocytogenes* ability to form biofilms in a low nutrient environment. This ability to adapt to low nutrient conditions may account for some of the differences in biofilm formation seen at 24 h, however by 96 h these variances were not apparent; this was also observed by Harvey et al.[30], indicating initial attachment within 24–48 h is key to FPE colonisation.

A potential influence on attachment and biofilm formation during the first 24 h is the growth rate of isolates. While it is well known there can be differences in growth rate between strains, in this study the ability to form biofilms was not associated with growth rate and doubling times at 14 or 37 °C which reflects the results of other published research. The independent nature of biofilm formation to growth rate has been reported in previous studies at temperatures reflecting FPE and also at 37 °C[24,31]. Lee et al.[32] noted less biomass was produced at 10 °C compared to biofilms at 37 °C, which were attributed to a lower growth rate and cell hydrophobicity at the cold temperature. Taylor and Stasiewicz[33] also found persistent strains did not display increased ability to grow in various energy sources and conditions with their ability to persist most likely strain-specific or the result of environmental conditions.

The extracellular matrix (ECM) is a necessary component of the biofilm structure and is composed of proteins, extracellular DNA, polysaccharides and exopolysaccharides and amyloid fibres, however the composition varies between species[34]. While *Listeria* is not known to be a producer of cellulose and poly-β-1,6-*N*-acetyl-D-glucosamine common amongst Proteobacteria which produce defined biofilms, it has been reported *Listeria* produces a novel EPS primarily composed of *N*-acetylmannosamine and galactose which is capable of binding congo red as an indicator[35,36]. Two phenotypes were present in the B10 group, pink indicative of some EPS production and translucent, negative for EPS production, with the production depended on the medium used for some strains. While the amount of EPS produced was not determined, the presence of EPS in *Listeria* has been linked with cell aggregation and increased tolerance to disinfectants and desiccation suggesting the B10 strains which are capable of producing EPS have increased ability to survive and persist within the FPE and display initial stages of biofilm formation[35]. EPS production was not associated exclusively with either faster or slower biofilm formation.

The *agr* system was initially described as a signalling peptide system in staphylococcal species[37], with orthologs *lam*[38] and *fsr*[39] being identified in *Lactobacillus plantarum* and *Enterococcus faecalis*, respectively, in addition to *L. monocytogenes*. The *agr* system is a peptide signalling communication four gene operon composed of *agrB*, a transmembrane protein which processes the propeptide encoded by *agrD* into a mature autoinducing peptide (AIP). The AIP is then exported into the extracellular environment until the concentration achieves a certain threshold, triggering the histidine kinase sensor *agrC* and activating the response regulator *agrA* which combine as a two-component system (*agrC-agrA*) applying transcriptional regulation including positive regulation[40–44]. The *agr* system has been shown in *L. monocytogenes* to be involved in invasion, pathogenicity and biofilm formation[45]. While this system has been shown to be linked to biofilm formation there is limited research on differences in expression between strong and poor biofilm producers at conditions reflecting the FPE. In this study, there was some statistical differences when comparing cells isolated from coupons to SM within either the fast

or slow group; however, there was no statistical difference in the expression of *agrD* between the fast isolates and the slow isolates. Gandra et al.[46] reported higher levels of the *agr* locus is expressed at 37 °C compared to 10 °C. In addition, they identified *agrBCD* genes are important for adhesion and the initial stages of biofilm formation particularly at 12 and 24 h. The results of this study support the upregulation of *agr* system elements in the early stages of attachment and early biofilm growth; however, expression appears to decline as the biofilm matures. In contrast, increased *agrD* expression was not observed in the planktonic cells of the spent media in this study at any of the timepoints measured, suggesting expression of this signal peptide is induced following attachment and initial biofilm formation, rather than planktonic growth, under the conditions tested.

To further investigate a genetic basis for the rapid colonisation phenotype, this study also examined the global transcriptomic response of *L. monocytogenes* during attachment and biofilm formation at 24 and 48 h under conditions reflective of the FPE. Four isolates from two STs (two isolates per ST) were chosen for RNA sequencing, with each ST cohort including one fast and one slow coloniser, to provide insights into variation in gene expression between fast and slow colonisation phenotypes. This included a lineage I and lineage II ST. Globally across strains metabolism and transport pathways were up regulated with variation of the pathways between strains. As a saprophyte, *L. monocytogenes* is exposed to varied, and at times limited nutrient sources and as such requires an extensive range of transport and metabolism mechanisms. Glaser et al.[47] identified 331 different transporter genes with 88 related to the phosphoenolpyruvate-dependent phosphotransferase systems (PTS) responsible for the transport and phosphorylation of various sugars and sugar derivates[48]. This extensive range of transporter genes is one of the largest known among bacterial species and allows *L. monocytogenes* to survive within a broad range of environmental and host conditions[47]. Furthermore, it allows for the bacterium to respond to any changes in its environment and adapt as necessary. In a few other bacterial species in which the biofilm genetic landscape has been eluded, PTS has been linked with the regulation of biofilm formation. In a study on *Klebsiella pneumoniae* biofilms, three genes encoding an enzyme II complex in PTS was found to increase eDNA and capsular polysaccharide production resulting in positive regulation of biofilm production[49]. Similarly, Houot and Watnick[50] found the *Vibrio* polysaccharide (*vps*) genes of *Vibrio cholerae* responsible for exopolysaccharide synthesis, were coregulated with PTS components and formation of multilayer biofilms were influenced by particular PTS sugars which activated the transcription of these *vps* genes. Unlike *V. cholerae*, the genetic determinants for *L. monocytogenes* biofilms are not well defined and comprise of a variety of genetic interactions, with most also having an established role in virulence and pathogenicity. In our study, various components of the PTS were upregulated at 24 h across the fast isolates, compared to the slow isolates, however there is limited research assessing how the PTS influences biofilm formation at conditions reflective of the FPE in *L. monocytogenes*. In this study, various elements of the PTS pathways up regulated in different strains further suggests colonisation differences are strain-specific and influenced by environmental conditions. Further research is required to determine if various components of the PTS are responding to its preferred nutrients as the result of the isolation environments selected in this study, or if the PTS have roles in the early stages of biofilm formation.

In conjunction with the PTS, various metabolic pathways associated with carbohydrates and sugars were also upregulated, including starch and sucrose metabolism at 24 h and amino and nucleotide sugar metabolism at 48 h across the fast isolates suggesting a switch to nutrient scavenging to initiate colonisation. Free glucose is often not readily available in the environment and as such alternative carbon sources are required. As mentioned previously, *L. monocytogenes* has an extensive transport system allowing this bacterium the ability to utilise various environmental carbon sources at times when nutrients are limited. Energy sources like cellobiose, lactose, lichenan, trehalose, maltose and their associated degradation products were all up regulated in this study as well as the 6-phospho-β-glucosidase, which suggests beta-glucosides are used by these strains. Taylor and Stasiewicz[33] found 97% of *L. monocytogenes* isolates tested (n = 95) grew in defined media supplemented with cellobiose, fructose or glucose however, lactose and sucrose were unable to support the growth of 79 and 72% of the isolates, respectively. An earlier study also reported fructose, mannose, cellobiose, trehalose were capable of supporting *L. monocytogenes* growth in the absence of glucose[51]. Mannose and trehalose supplementation has also been shown to increase biofilm development over 12 days[52]. The results of this study suggest a global upregulation of diverse metabolic pathways under nutrient limited, low temperature conditions may facilitate adaptation and maximised nutrient scavenging, contributing to initiation of a biofilm lifestyle and persistence of *L. monocytogenes* under similar conditions founds in FPE.

Amino sugar metabolism has been connected to energy production and biosynthesis of cell wall peptidoglycan and teichoic acids[53,54]. Key enzymes of the amino sugar and nucleotide sugar pathway up regulated in this study at 48 h includes *N*-acetylglucosamine-6-phosphate deacetylase (*nagA*) and glucosamine-6-phosphate deaminase (*nagB*), indicating at 48 h under conditions reflective of the FPE the fast isolates are undergoing an increase in biomass through the biosynthesis of peptidoglycan cementing their ability to survive in the FPE. N-Acetylglucosamine (GlcNAc) is an abundant carbon and nitrogen source found throughout the environment (as a chitin monomer) and as part of bacterial cell wall peptidoglycan[55]; it has been reported *L. monocytogenes* can turnover between 30–50% of its cell wall peptidoglycan every generation[53]. The deacetylation of N-acetylglucosamine-6-phosphate by NagA into glucosamine-6-phosphate and acetate is a part of peptidoglycan degradation and thus cell wall recycling[53]. Glucosamine-6-phosphate can be further transformed into fructose-6-P by NagB for energy production through the glycolysis pathway[54]. An additional key enzyme in peptidoglycan biosynthesis is UDP–*N*-acetylglucosamine (UDP-GlcNAc) 1-carboxyvinyltransferase (MurA) responsible for the addition of enolpyruvyl from phosphoenolpyruvate to UDP-GlcNAc[56]. The paralogue version, *murZ* was up regulated in this study. The combination of *nagA*, *nagB* and *murZ* suggests cells were possibly undergoing cell wall synthesis to increase biofilm mass. This adaptation again suggests a global switch to nutrient scavenging and biomass increase is a central strategy to the initial colonisation of FPE by *L. monocytogenes*.

Three genes involved in pyrimidine metabolism and alanine, aspartate and glutamate metabolism pathways were upregulated at 48 h in the ST101 comparison. The genes observed related to pyrimidine metabolism are

involved in de novo synthesis of uridine-monophosphate (UMP) starting from glutamine and include, *pyrAa*, carbamoyl-phosphate synthase small chain, glutamine-utilizing subunit of carbamoyl-phosphate synthetase, similar to the *carA* of the same role in *E. coli*, *pyrB*, catalytic subunit of aspartate carbamoyltransferase and *pyrC*, dihydroorotase[57,58]. A study by Pisithkul[59] into biofilm development of *Bacillus subtilis* found expression of pyrimidine synthesis enzymes and other nucleotides and biosynthetic precursors peaked at 16 h then declined slowly for the remainder of the study. In another study, Hingston[60] identified *pyrAaBC* genes were up regulated at 4 °C during the transition to stationary phase. De novo synthesis of UMP has been linked to biofilm formation and production of cellulose and curli fimbriae in *E. coli* through transcription of the *csgDEFG* operon[61]. While the *pyr* operon has not been linked to biofilm formation in *L. monocytogenes*, our results suggest it may be linked in some way, however further research is required.

Interestingly, the cobalamin biosynthesis pathway and genes involved in the cobalamin-dependent gene cluster (CDGC) were also identified as being overexpressed at 24 h in isolate 7453^{F}. Cobalamin genes are responsible for vitamin B_{12} biosynthesis which is required as enzyme cofactors for various metabolic process particularly during the metabolism of ethanolamine and 1,2-propanediol as carbon and nitrogen energy sources[62]. Cobalamin biosynthesis can occur during aerobic respiration with *cob* genes or during anaerobic respiration utilising *cbi* genes[63]. In this study more *cbi* genes (compared to *cob*) from the Cobalamin anaerobic pathway were upregulated, in addition, genes involved in ethanolamine (FC range 3.2–6.8) and propanediol utilisation (FC range 2.03–4.2) were also up regulated and have been shown to be activated during stressful, competitive conditions and during cold temperatures[64–67]. In a transcriptomic study by Hingston and colleagues[60], they reported an increase in genes associated with ethanolamine utilisation at multiple growth phases at 4 °C. The upregulation of the genes from the CDGC may reflect *L. monocytogenes* is experiencing stress as a result of the low temperature and limited nutrients within the biofilm state. These systems facilitate greater flexibility in nutrient scavenging and utilisation through metabolism of alternative substrates, which is critical for survival when optimal nutrients are unavailable or competition with other microbial species is ongoing[66,68].

Ribosomes are essential protein synthesising components that are involved in sensing and responding to their environmental conditions[69]. In prokaryotes they are composed of a 50S large subunit, where the peptide bonds are formed, and a 30S small subunit that binds the messenger RNA, creating a 70S ribosome[70]. In this study, a variety of ribosomal proteins were upregulated with a majority being the large subunit. Each subunit contains 30 and 20 ribosomal proteins (R-proteins) designated L or S for the 50S or 30S subunits respectively. R-proteins have various roles including translation, assembly, cell proliferation and cellular differentiation with some of these roles essential for survival[71]. In this study, up regulation of ribosome proteins may reflect the global level of transcription and translation is higher under conditions reflective of the FPE due to multiple sub-optimal factors at play, however, there is limited research on the R-proteins in *L. monocytogenes* to be able to elude to more specific roles in this study.

Within isolate 7453^{F} and ST101 at 24 h there was a considerable number of differentially expressed prophage genes expressed suggesting prophage genes may influence the initial stages of colonisation. Over 500 *L. monocytogenes* bacteriophages have been identified, with a large portion being temperate phages capable of inserting themselves into the bacterial chromosome[72]. Temperate phages have been linked with providing increased fitness to host bacterial strains[73]. A common temperate listeria phage A118 has been shown to insert itself into the competence protein K (*comK*). A study by Verghese et al.[28] showed meat and poultry isolates containing the *comK* prophage were capable of growing to higher cell densities with the authors suggesting its insertion allows strains to adapt to niches which influence their colonisation and persistence in FPE. In an earlier study on *E. coli* K12 strains containing cryptic prophage, they found increased fitness against osmotic, oxidative and acidic stress and increases in biofilm formation and growth[73]. While there have been limited studies reporting lab based phenotypic benefits of *L. monocytogenes* isolates containing prophages, the up regulation of prophage genes in this study opens the possibility they may play some role in either low nutrient adaption, attachment or biofilm formation. In this study, phage A118 is inserted into the *comK* gene of isolate 7453^{F} suggesting the presence of phage A118 may influence this isolate's ability to rapidly colonise the FPE by increasing cell density and withstanding the suboptimal conditions found in FPE.

The DE of regulators and repressors involved in stress response and biofilm formation can be indicators of which stress systems are responding to sub-optimal conditions, it is important however to note that it is not one particular regulator being induced rather a variety of different regulators and repressors indicating the complex nature of the FPE and the overlap in stress response and virulence related genes and systems. In this study, *recA/lexA*, responsible for DNA repair and activation of the SOS response during stressful conditions in *L. monocytogenes*, was upregulated in three strains and one strain respectively. The SOS response is required for bacterial adaptation, diversification and pathogenesis in a majority of species and has been reported to be required for biofilm formation in *E. coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Mycobacterium tuberculosis*[74]. Van der Veen et al.[75] showed *recA* also influences genetic variability through mutagenic repair during continuous flow biofilms. The mutagenic repair of DNA may be critical for biofilm formation and resistance to stress conditions along with the development of disinfectants and antibiotic resistance[76]. The upregulation of *recA* and *lexA* is indicative of the stress conditions experienced in this study from the low temperature and limited nutrients utilised.

The presence of flagella and motility related genes have been shown to be involved in initial attachment stages and subsequent biofilm formation and colonisation in *L. monocytogenes* on various processing environment and produce surfaces[77,78]. In isolate 7538 the *degU* response regulator was upregulated. Previously, *degU* has been associated with flagella biosynthesis, chemotaxis, attachment and biofilm formation[79,80]. Gueriri et al.[80] suggests that *degU* may play a role in biofilm formation that is distinct from the essential role it plays in regulating flagella synthesis. In addition, Pieta et al.[81] showed *degU* was equally or significantly increased at 7 °C when compared with 37 °C. Therefore, the upregulation of *degU* is suggestive of cells undergoing biofilm formation,

as strain 7538 was a slow biofilm former it may be the motility of cells at lower temperatures may be regulated later compared to fast biofilm formers.

The *mogR* gene is the transcriptional repressor of flagella motility at all temperatures and specifically at temperatures relevant for infection[82]. Cordero et al.[83] reported strains which demonstrated faster growth rates at low temperatures displayed reduced flagella expression to conserve energy yet remain prolific. Isolate 7545 displayed *mogR* expression suggesting flagella motility was reduced potentially as a metabolic function to save energy and continue multiplying in the limited nutrients and low temperature conditions used in this study.

The class three stress gene repressor, *ctsR*, which regulates class three heat shock genes was upregulated in isolate 7538. In addition, *ctsR* has also been indirectly linked with virulence, motility gene expression and has been shown to be coregulated with other regulators including *sigB*, *sigH*, *hrcA* and *prfA*[60,84–86]. The general stress response gene, *sigB*, was also upregulated in this isolate. The alternative sigma factor β (*sigB*) is a major stress response regulator of general stress response and class II stress genes which are required for various stress related conditions, including cold, acidic, osmotic, oxidative stress and high pressure processing[87,88]. *SigB* has been shown to be required for the biofilm mode of life in both, static and continuous flow biofilms[87], in addition there are reports it is required during starvation survival in low nutrient environments[89]. In this study, suboptimal conditions used were to reflect the stressful climate in the FPE therefore the upregulation of *ctsR* and *sigB* is an adaptive mechanism *L. monocytogenes* most likely employees to survive within the FPE.

The *fur* gene is required to regulate intracellular levels of iron which is an essential cofactor required for many important enzymatic roles in bacterial cells[90,91]. *Fur* regulation has been linked with oxidative stress response and protection against ROS damage[92,93]. In addition, in low iron environments f*ur* regulation plays a significant role in sequestering iron within increased levels of *fur* transcription reported in these environments[92,94]. The upregulation of f*ur* may be indicative of low iron levels as a result of the limited nutrient environment or cold stress conditions utilised in this study. Further, *fur* regulation has been linked with metabolic function in bacteria[94]; the conditions in this study resulted in a diverse range of metabolic systems upregulated and therefore the upregulation of *fur* may be reflective of the metabolic state cells in biofilm are undergoing.

In this study we aimed to replicate elements present in the FPE to determine their influence on the colonisation by *L. monocytogenes*. Although the results obtained provide beneficial insight into our understanding of this subject, it was not without its drawbacks. The multiple factors analysed in combination have provided some generalised understanding and identified baseline research against more isolates is required. For the ST comparison the isolates were not isogenic strains however based upon the average nucleotide identity (99.91% for isolates 7453 and 8116 and 99.90% for isolates 7545 and 7538) the isolates selected were considered suitable for comparison purposes. In addition, the expression data for a small number of genes which are not shared between the comparison isolates may be absent as a result of using non-isogenic strains.

Concluding remarks

L. monocytogenes isolates are a concern for public health due to their ability to colonise and persist in FPEs. The economic and brand reputation for a food processing company can be substantial should *L. monocytogenes* strains contaminate RTE food products and cause listeriosis. This study looked at various factors which may influence *L. monocytogenes* ability to colonise a processing facility. We demonstrated that the ability to form biofilms was different from strain to strain and was not linked to differences in growth at conditions reflective of the FPE, nor cellulose or curli expression as identified in other species like *E. coli* and *Salmonella*. While there were also no specific genes identified by the GWAS, interestingly the global transcriptome indicated metabolic mechanisms were upregulated, suggesting the species utilizes its wide metabolic and transport repertoire to initiate a rapid adaptation to nutrient limited conditions. This is then coupled with upregulation of genes involved in the production of cell structural components for biofilm expansion, with upregulation of the *agr* system in the initial attachment and biofilm growth. Colonisation is likely aided through environmental factors like hard to clean and sanitise niches, and genetic determinants like the ability to form biofilms and attach in suboptimal conditions, our knowledge of *L. monocytogenes*' ability to persist and survive in the FPE requires further exploration, as this knowledge will be necessary in order to prevent and mitigate contamination.

Methods

Bacterial isolates, culturing conditions and subtyping. A total of 52 *L. monocytogenes* isolates from 12 sequence types (ST, up to 5 isolates per ST) representative of multi-locus sequence types commonly associated with FPEs in previous analyses[95,96], and previously isolated from a variety of food-related sources (i.e., dairy, meat, vegetable, mixed food and environment; Supplementary Table S1) were chosen. Isolates selected each possessed unique pulsed field electrophoresis pulsotypes, to increase strain variance. Isolates were removed from − 80 °C storage and resuscitated on Brain Heart Infusion (BHI, CM1136, Oxoid, UK) agar at 37 °C for 24 h.

Stainless steel coupons. Stainless steel (SS) coupons of grade 304, mill finish (5 mm diameter by 0.9 mm thick; surface area 0.53 cm^2) were utilized. Coupons were cleaned in a solution of 3% sodium hydroxide (Sigma-Aldrich, 72068, Australia) for 20 min, then 0.1% peracetic acid (Oxysan, C16620, Australia) for two minutes. Coupons were rinsed with sterile water three times between washes and then sterilised in the autoclave.

Biofilm formation analysis. *L. monocytogenes* isolates were grown for 18 h (± 1 h) in BHIB at 37 °C. A high throughput biofilm screening method, previously developed[97], was used to determine the fastest and slowest biofilm forming isolates. Briefly, microtiter plate wells containing SS coupons were inoculated aseptically with 100 µL of 10^3 CFU/mL in 1:10 diluted BHI (dBHI) and incubated at 14 °C for 24, 48, 72 or 96 h (± 1 h) statically. After the appropriate incubation period the spent medium were removed, SS coupons were transferred

Primer set	Oligonucleotide sequence 5′→3′
agrD-F	CAGTTGGTAAATTCCTTTCTAGAAAAC
agrD-R	TTTTCACAAATGGACTTTTTGGTTCG
rpoB-F	TGGGGCAGAACGTGTTATCG
rpoB-R	CCCACGGTTAGGGATGACAG

Table 5. Real time-PCR primer sequences designed for this study.

to a sterile microtiter plate and underwent three rinses with sterile water. Coupons were sonicated in wells with Maximum Recovery Diluent (MRD; Oxoid, Thermo Scientific, Australia) for 5 min then 100 µL was serial diluted and plated onto BHI agar (BHIA) for enumeration at 37 °C for 24 h prior to counting. Two biological replicates each with two technical replicates were performed on all 52 isolates, with an additional two biological replicates, again with two technical replicates, performed on 10 isolates. These 10 isolates comprised those with the fastest ($n = 5$) or slowest average biofilm cell numbers after 24 h (referred to as the B10 isolates).

Growth rate determination. Growth curves were constructed for the planktonic B10 isolates at 37 °C in undiluted BHI and at 14 °C in dBHI. For the growth curves, a single colony of each B10 isolate was inoculated in 5 mL BHI at 37 °C at 150 rpm for 18 h (± 1 h). For the 37 °C growth curve, 200 µL of a 1:200 dilution was aliquoted into a 96 well microtiter plate and growth was monitored for 12 h at OD_{600} using an EON microplate spectrophotometer Gen5 (BioTek, Australia). For the 14 °C growth curves, a 1:200 dilution of the 18 h (± 1 h) culture into dBHI was aliquoted into conical flasks and growth was monitored every 4 h until timepoint 15 h when growth was measured every 2 h at OD_{600} for 31 h. Maximum growth rate (µ) and doubling times (t_d) (2) were determined during the exponential growth phase using the equations: $\mu = (\ln OD_2 - \ln OD_1)/(t_2 - t_1)$ and $t_d = 0.693/\mu$, respectively, where ln refers to the natural logarithm, OD_2 is late exponential phase OD, OD_1 is early exponential phase, t_2 is time in minutes for OD_2 reading and t_1 is time in minutes for OD_1 reading, t_d is doubling time and µ is growth rate.

EPS production. Exopolymeric substance analysis was performed as follows: lysogeny broth (LB) agar without salt supplemented with 40 µg/mL Congo Red (CR) and 20 µg/mL Coomassie Brilliant Blue (CBB) was spotted with 5 µL of the 18 h (± 1 h) culture and incubated at 14 °C for 48–72 h and 37 °C for 24–48 h. For the CR assay, 18 h (± 1 h) cultures were grown in LB without salt and Muller Hinton broth at 37 °C and 150 rpm.

Large batch biofilm formation. The biofilm process was upscaled for RNA extractions at 24 and 48 h for the B10 isolates. The biofilm process followed the initial screening experiment with the following changes: two coupons (15 mm × 15 mm × 0.55 mm) were used per isolate time point, coupons were transferred to a new 70 mL yellow cap container for three washes with DEPC-treated molecular grade water prior to biofilm removal with a cell scraper then sonication for 5 min. Cell scrapers were vortexed briefly for 10 s then pulse vortexed five times to remove any attached cells. Cells were pelleted at 7000×*g* for 10 min.

Total RNA extraction. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Australia) with the following adjustments: 25 mL of spent medium (SM) was collected from each time point and 1 mL of *Escherichia coli* DH5α was added as a carrier to assist in centrifugation of pellet. For the coupons, 2 mL of *E. coli* DH5α was used. RNA stabilisation was performing using a 5% phenol ethanol solution as per Bhagwat et al.[98]. Enzymatic lysis of cells consisted of 100 units of mutanolysin (Sigma-Aldrich/Merck Australia) for 15 min in a 22 °C water bath followed by 20 µL of 20 mg/mL Proteinase K for an additional 15 min. During RNA purification the spin column was washed twice with Buffer RW1. RNA yields were assessed on a Nanodrop device ND-1000 (Nanodrop, Thermo Fisher, Australia) and RNA quality assessment was performed on 2200 TapeStation System (Agilent, Australia) using high sensitivity RNA screen tapes. Samples were stored at − 80 °C until reverse transcriptase and RNA sequencing.

Genome wide association study. A genome wide association study (GWAS) was performed using the R package treeWAS[99] to identify genetic variants potentially responsible for variances in the biofilm phenotype at 24 h utilising a phylogenetic method accounting for population structure and recombination. Kchooser and Ksnp3[100] was used to generate the optimal kmer value and core SNP matrix file from the biofilm isolates genome sequences.

Real time qPCR. DNase treatment and cDNA synthesis were performed on 1 µg of RNA using the iScript gDNA clear cDNA synthesis kit (Bio-rad, Australia) as per manufacturer's protocol. Real time qPCR (RTi-qPCR) was performed targeting the propeptide *agrD* as the gene of interest and *rpoB* as the housekeeping normalisation gene on the AriaMX Real-time PCR System (Aglient). Primer sequences were designed using primer3 in Geneious (2018) (Table 5). RTi-qPCR amplification was performed in 20 µL reactions with the mix containing 10 µL iTaq universal SYBR green Supermix (Bio-rad), 1 µL forward and reverse primers, 6 µL molecular grade water (Sigma-Aldrich) and 2 µL cDNA. PCR conditions were as follows: 3 min at 95 °C followed by 40 cycles at 5 s at 95 °C and 45 s at 60 °C. Assays included a non-template control and non-reverse transcriptase for sample

control with three biological replicates each with three technical replicates. Relative expression was determined using the $2^{-\Delta\Delta}$CT method[101]. Stata (Stata 15.1, StataCorp, College Station, Texas, USA) was used for statistical and data analysis. The nonparametric Wilcoxon rank sum test was performed on independent samples and the Wilcoxon signed rank test was performed on paired samples (p value < 0.05).

RNA sequencing and analysis/transcriptomics. Four isolates from two STs which had an isolate in both the fast and slow biofilm formation groups were chosen for RNA sequencing. Total RNA extracts for sequencing were measured using the Qubit RNA high sensitivity kit (Thermo Fisher) and RNA extracts were sequenced by Western Sydney University Next Generation sequencing facility (NSW, Australia). Zymo-Seq RiboFree Total RNA Library Prep kit was used for rRNA depletion following the manufacturer's protocol. Depleted RNA samples were clustered on cBot and sequencing was performed as 2 × 125 bp paired end TrueSeq Cluster kit v4 and HiSeq SBS v4 kit on the Illumina HiSeq 2500 platform.

Sequence alignment was performed in Galaxy Australia[102] using the following tools: reads were mapped to each isolates draft genome sequence using BWA-MEM (Galaxy v0.7.17.1)[103], JBrowse genome browser was used to view the mapped reads (Galaxy v1.16.4 + galaxy3)[104], SAM/BAM to count matrix using HTSeq code (v0.5) was used to produce differential gene expression (DGE) count matrices. Gffread (Galaxy v0.11.6.0) was used to convert .gff3 files from the Patric database[105,106] to .gtf files for count analysis. The $\log_2$ counts per million for the DGE count matrix were determined by Voom/Limma in Degust (v4.1.1)[107]. Individual isolate comparisons consisted of 7453 24 h with 7453 48 h, 7545 24 h with 7545 48 h, 8116 24 h against 8116 48 h and 7538 24 h and 7538 48 h. For ST comparison, analysis was performed by comparing the two isolates from within the same ST at the same timepoint. The ST101 24 h comparison consisted of isolates 7453 24 h and 8116 at 24 h, the ST101 48 h comparison was against 7453 48 h and 8116 48 h. The ST2 24 h analysis was between 7545 24 h and 7538 24 h and the ST2 48 h comparison contained 7545 48 h and 7538 48 h. The draft genome sequences of 7538 and 8116 was used as the reference genome for ST2 and ST101 respectively. Functional annotation was performed with Eggnog mapper v2 (v2.0.0) using Listeriaceae as the taxonomic scope and gene ontology from experimental evidence only with all other fields default. The functional annotation was matched to differentially expressed genes (DEGs) using Excel and were analysed based upon their clusters of orthologous groups (COG) category with tRNAs allocated to COG category J and hypothetical proteins and DEGs with no COG category assigned to category S to include in the analysis. Overexpressed protein pathways were determined using STRING (v11)[108,109] by submitting the amino acid sequences for all the DEGs (FDR < 0.01 and $\log_2$ FC) with *L. monocytogenes* EGD-e as the organism reference. Statistical significance was determined for overexpressed protein pathways with a false discovery rate (FDR) < 0.01 and absolute log fold change (logFC) of ≥ 2 for 24 h vs 48 h samples. Differentially expressed regulatory genes were determined utilising an FDR < 0.05. Regulatory genes of interested were determined based upon the conditions utilised in the simplified model biofilm system and reflected the isolates potential systems/pathways used to respond to these conditions and included the following genes: *ctsR, hcrA, lexA, perR, codY, agrA, sigB, fur, recA, mogR, degU, virR* and *prfA*.

Data availability

The raw sequencing data were deposited at the NCBI Sequence Read Archive under Bioproject No. PRJNA715821.

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Author contributions

J.G., P.S.C. and E.F. conceived and designed the study. J.G. performed the experiments. Data was analysed by J.G., E.F., P.S.C., J.P.B., M.K., C.K. and S.F. J.G., P.S.C. and E.F. drafted the manuscript. All authors corrected and approved the manuscript.

Competing interests

The authors declare no competing interests.

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Characterisation of *Listeria monocytogenes* food-associated isolates to assess environmental fitness and virulence potential

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ABSTRACT

The ability of *Listeria monocytogenes* isolates to survive within the food production environment (FPE), as well as virulence, varies greatly between strains. There are specific genetic determinants that have been identified which can strongly influence a strains ability to survive in the FPE and/or within human hosts. In this study, we assessed the FPE fitness and virulence potential, including efficacy of selected hygiene or treatment intervention, against 52 *L. monocytogenes* strains isolated from various food and food environment sources. Phenotypic tests were performed to determine the minimum inhibitory concentration of cadmium chloride and benzalkonium chloride and the sensitivities to five clinically relevant antibiotics. A genomic analysis was also performed to identify resistance genes correlating to the observed phenotypic resistance profiles, along with genetic determinants of interest which may elude to the FPE fitness and virulence potential. A transposon element containing a novel cadmium resistance gene, *cadA7*, a Tn*916* variant insert in the hypervariable *Listeria* genomic island 1 region and an LGI2 variant were identified. Resistance to cadmium and disinfectants was prevalent among isolates in this study, although no resistance to clinically important antimicrobials was observed. Potential hypervirulent strains containing full length *inlA*, LIPI-1 and LIPI-3 were also identified in this study. Cumulatively, the results of this study show a vast array of FPE survival and pathogenicity potential among food production-associated isolates, which may be of concern for food processing operators and clinicians regarding *L. monocytogenes* strains colonising and persisting within the FPE, and subsequently contaminating food products then causing disease in at risk population groups.

1. Introduction

Listeria monocytogenes is a saprophyte and a human pathogen. *L. monocytogenes* can cause the severe disease, listeriosis, in at risk populations that includes the elderly, immuno-compromised, pregnant women and neonates with an associated mortality rate of almost 30% (Havelaar et al., 2015; Todd and Notermans, 2011). *L. monocytogenes* can enter the food production environment (FPE) through multiple vectors, such as the introduction of contaminated raw ingredients, or the ingress of staff or pests carrying the bacterium, which can then progress to the colonisation of the production environment and subsequent cross contamination of food products, particularly ready to eat (RTE) items (Camargo et al., 2017). Although not all strains which enter the FPE will go on to cause listeriosis, there are a variety of genetic and phenotypic traits/mechanisms which can indicate a strains ability to survive or cause disease. An understanding of the various factors influencing colonisation, survival and pathogenicity is thus important.

Within the FPE there are intrinsic (food-related) and extrinsic (intentionally applied to reduce microbial contamination and spoilage) stress factors utilised to control *L. monocytogenes* strains including high osmolarity, temperature and pH, disinfectants, sanitisers and episodes of desiccation (Jordan et al., 2018). *L. monocytogenes*' ability to survive various processing/hurdle technologies influences its ability to colonise and persist in the FPE, making it an important foodborne pathogen.

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Survival throughout the FPE can result in cross contamination of products like RTE foods, which are considered high risk products due to the lack of further cooking prior to consumption, and common vehicles for listeriosis outbreaks (Camargo et al., 2017; Jordan et al., 2018). Importantly, it has been suggested conditions present within the FPE may promote the development of survival strategies like cross protection and interconnectedness between tolerance or resistance to multiple stressors (Bucur et al., 2018). At refrigeration temperatures, or lower ambient temperatures used in many FPEs, an increase in tolerance of associated *L. monocytogenes* strains to cold temperatures, osmotic and oxidative stressors has been noted (Manso et al., 2020; Schmid et al., 2009). Recently identified genomic islands, stress survival islet (SSI)-1, and SSI-2, are responsible for tolerance to acid, salt, bile, gastric, alkaline and/or oxidative stress, further highlighting the diverse genomic arsenal which supports niche adaptation, survival and persistence of *L. monocytogenes* in the FPE (Harter et al., 2017; Ryan et al., 2010). In addition, the presence of plasmids and prophage elements also increases an isolates fitness within the FPE (Schmitz-Esser et al., 2015; Verghese et al., 2011).

An understanding of the pathogenic potential of *L. monocytogenes* strains isolated from the FPE, as well as their resistance to antibiotics, is also important. The listerial infection cycle is the result of several essential virulence factors, predominately a six gene virulence cluster also known as *Listeria* pathogenic island 1 (LIPI-1) (Hadjilouka et al., 2016; Karthikeyan et al., 2015; Luo et al., 2013; Osman et al., 2020; Poimenidou et al., 2018). In addition, there are other important genes involved in virulence, including *inlA* and *inlB*, which are required for initial invasion (Autret et al., 2001). However, not all isolates in the FPE contain functional virulence genes, with mutations in key virulence genes like *prfA* or *inlA* resulting in a reduced pathogenic potential (Miner et al., 2008; Nightingale et al., 2008). Determining the presence and the degree of diversity can provide an overview of the pathogenicity potential of transient or persistent isolates within the FPE (Poimenidou et al., 2018). Differences in virulence is an important public health concern as highly virulent strains can be associated with outbreaks and severity of illness, and therefore the sensitivity to clinically relevant antibiotics is also required. Importantly, resistance in *L. monocytogenes* isolates have been increasingly reported, along with the presence of genetic determinants being identified for various classes of antibiotics, some of clinical relevance (Grayo et al., 2008; Rakic-Martinez et al., 2011; Wilson et al., 2018); therefore continued surveillance of antibiotic sensitivity is required. Typically, the pathogenic potential of clinical isolates is looked at, with less studies considering food-isolated *L. monocytogenes* ability to both cause disease, in addition to the carriage of genes that may support their survival within the FPE. The aim of this study was to characterise *L. monocytogenes* isolates from food and food-environment sources using phenotypic and genomic methods to determine their ability to survive within the food production environment, their potential to cause infection and their susceptibility to frequently used antibiotics in the treatment of listeriosis.

2. Materials and methods

2.1. Bacterial strains, isolation and molecular characterisation

A total of 52 *L. monocytogenes* isolates from 12 sequence types (ST, up to five isolates per ST) representative of multi-locus sequence types commonly associated with the food chain were chosen from a variety of sources (dairy, meat, vegetable, mixed food and environment; Supplementary Table 1) across a span of 18 years (1998 to 2016). Isolates selected each possessed unique pulsed field gel electrophoresis pulsotypes to increase strain variance (data not shown). Isolates were stored in a −80 °C freezer, and resuscitated on Brain Heart Infusion (BHI, Oxoid, Australia) agar at 37 °C for 24 h, prior to experimental manipulations.

2.2. Phenotypic characterisation

2.2.1. Antimicrobial sensitivity

The sensitivity of *L. monocytogenes* strains to five antibiotics used for the treatment of listeriosis was determined on Muller Hinton Agar (Oxoid, Australia) supplemented with 5% (v/v) defibrinated sheep blood (MHSBA; Thermo Fisher Scientific, Australia). Ciprofloxacin, trimethoprim/sulfamethoxazole and penicillin G (0.002–32 μg/mL) along with gentamicin and amoxicillin (0.016–256 μg/mL), were assessed. Bacterial suspensions adjusted to 0.5 McFarland in maximum recovery diluent (MRD) were inoculated onto MHSBA using a sterile swab in three directions and incubated at 37 °C for 24–48 h. The minimum inhibitory concentration (MIC) for each antibiotic was assessed using Etest strips (Biomerieux, Australia) and MIC values were interpreted according to breakpoints provided by the Clinical Laboratory Standards Institute (CLSI) (CLSI, 2014, 2016) or European Committee on Antimicrobial Susceptibility Testing (EUCAST) (EUCAST, 2019) or the literature (Noll et al., 2018) for *L. monocytogenes* where available; or staphylococcal species MICs were used in the absence of breakpoints from the aforementioned sources. The MIC value for susceptibility was defined as ≤1 μg/mL for amoxicillin, ≤1 μg/mL for gentamicin and ciprofloxacin, ≤2 μg/mL for penicillin and ≤0.064 μg/mL for trimethoprim/sulfamethoxazole. The control strains used were *S. aureus* ATCC 29213 and *Streptococcus pneumoniae* ATCC 49619.

2.2.2. Disinfectant and heavy metal sensitivity

Benzalkonium chloride (BC) (Sigma Aldrich, Australia) was used to determine sensitivity of *L. monocytogenes* strains to an important industrial quaternary ammonium compound disinfectant, using a broth microdilution method with the following modifications: briefly, *L. monocytogenes* strains were grown overnight in Mueller Hinton broth (MHB) and diluted to ~10^3 CFU/mL, then 190 μL was inoculated into 96 well microtitre plate. For each BC stock concentration, 10 μL was added to the microtitre plates to achieve final concentrations of 50, 40, 30, 20, 10, 5, 2.5, 2, 1.5, 1 and 0.5 μg/mL. All plates were incubated at 37 °C for 24 h. Growth was monitored immediately following inoculation (T0) and again at 24 h (T24) at OD_{600} using EnSpire™ multilabel plate reader 2300 (PerkinElmer, Singapore). The T24 reading was subtracted from T0 to determine if isolates were capable of growth. The bacteriostatic or bactericidal effect of BC was tested for any isolate with growth under OD_{600} 0.1 with 10 μL spotted onto Brain Heart Infusion agar (BHIA) and incubated at 37 °C. After 24 h plates were checked for growth or no growth. A minimum of two biological replicates were performed.

Cadmium chloride ($CdCl_2$) was used to determine *L. monocytogenes* sensitivity to the heavy metal cadmium, using a previously optimized method (Ratani et al., 2012). Mueller Hinton agar (Oxoid, Australia) was supplemented with $CdCl_2$ (Sigma Aldrich, Australia) at the following final concentrations: 10, 35, 40, 70, 140 and 150 μg/mL. Isolates were grown overnight on BHIA and a 1 μL loopful was inoculated into 2 mL phosphate-buffered saline (PBS) and vortexed until completely suspended. The PBS and isolate solution were streaked with a cotton swab onto the $CdCl_2$ plates in three directions and incubated at 37 °C. Plates were visually assessed for growth or no growth after 48 h. Two biological replicates were performed.

2.3. Genomic characterisation

Genomic characterisation was predominately performed in Geneious (2020). Genes of interest were downloaded from NCBI with searches performed in Geneious using Megablast or tblastn, with positive results for hits displaying >85% query coverage and pairwise identity. A phylogenetic tree was created based upon raw reads using Snippy and Snippy-core (Seemann, 2015) in Galaxy Australia (Jalili et al., 2020) utilising the genbank file of isolate 7943 as the reference genome and reconstructed with RAxML (v8.2.4) (Stamatakis, 2014), utilising substitution model 'GTRCAT' and the remainder with default parameters.

2.3.1. Genetic determinants of virulence potential and FPE stress survival

The LIPI-1 virulence cluster and a selection of genetic determinants identified in the literature were chosen to assess the potential of the isolates to survive various stress conditions encountered within the FPE, and the potential to cause disease should a contaminated food product be consumed (Table 1). The protein or gene was downloaded from NCBI and a BLAST search of the genetic determinants occurred in Geneious utilising the above criteria. EasyFig 2.2.5 (Sullivan et al., 2011) was used to visualise gene comparisons.

2.3.2. Antimicrobial and virulence gene databases

Mass screening of acquired antimicrobial and virulence genes were performed using Abricate in Galaxy (v1.0.1) (Seemann, 2016) against the associated databases NCBI AMRFinder Plus (Feldgarden et al., 2019), CARD (Alcock et al., 2020; Jia et al., 2017), ARG-ANNOT (Gupta et al., 2014), Resfinder (Zankari et al., 2012) and VFDB (Chen et al., 2016).

2.3.3. Mobile Genetic Elements characterisation

Plasmids were identified using PlasmidFinder 2.1 against the Gram-Positive database (Carattoli et al., 2014). Prophage elements were identified using the online platform PHASTER (Arndt et al., 2016; Zhou et al., 2011). Draft nucleotide sequences were utilised for both analyses. Confirmation of plasmid and prophage results was performed in Geneious (2020), through contig interrogation and read-mapping. Comparison of closed plasmids was visualised using BRIG (Alikhan et al., 2011), with the following combinations: ST8 plasmids with pLM1686 as the reference plus an additional section from p7922 from this study, ST121 and ST321 utilising pLM6179 for reference and ST3, ST9, ST155 and ST204 were compared to pN1-011A and pR479a plasmids.

2.3.4. Data availability

Draft genome sequences for strains from this project have been deposited in the NCBI genome database or sequence read archive under BioProjects: PRJNA725037, PRJNA320339, PRJNA377767, PRJNA295145, PRJNA295464, PRJNA692370 and PRJNA422580; accession numbers are displayed in Supplementary Table 1.

Table 1
Plasmids identified among isolates in this study.

Isolate	MLST	Plasmid		
		Closed/open	Size (bp)	GC (%)
7514	3	Open	59,826	35.2
7547	3	Open	32,307	36.1
7553	3	Closed	4176	34.1
7583	3	Open	56,146	36
7922	8	Closed	88,290	36.5
8112	8	Closed	79,144	36.7
8120	8	Open	85,394	36.9
8124	8	Closed	79,180	36.7
8117	9	Closed	25,550	36.5
8118	9	Closed	25,550	36.5
8119	9	Closed	25,550	36.5
8123	9	Open	49,141	36.3
8129	9	Open	49,281	35.8
7425	121	Closed	62,207	36.5
7475	121	Open	60,666	36.7
7495	121	Open	62,191	36.5
7987	121	Closed	60,923	36.6
8113	121	Closed	62,207	36.5
7533	155	Open	64,751	38.1
7920	155	Closed	77,756	37.5
7921	155	Closed	80,184	37.4
7488	204	Open	48,687	37.4
7919	204	Closed	38,191	37.3
7929	204	Open	91,345	37.7
7943	321	Open	66,904	36.5
8126	321	Open	60,124	36.7

3. Results

3.1. Genomic composition of L. monocytogenes isolates

An overview of the genomic composition of the 52 *L. monocytogenes* isolates included in this study is shown in Supplementary Table 2. The draft genome sizes ranged between 2.61 and 3.08 Mb, with the GC percentage between 37.7 and 38.1%. The number of coding DNA sequences ranged from 2668 to 3165.

3.2. Cadmium chloride and benzalkonium chloride phenotypes

The sensitivity of the 52 *L. monocytogenes* strains to various concentrations of BC and $CdCl_2$ is shown in Fig. 1. When assessed against BC only two isolates (7544 and 7546) were unable to grow at the lowest concentration (0.5 μg/mL), however when subsequently spotted onto BHI agar they were able to produce colonies indicating BC at 0.5 μg/mL had a bacteriostatic effect on these two isolates. Assessment against varying concentrations of $CdCl_2$ resulted in 10 isolates unable to grow at the lowest concentration with the remainder of the isolates growing at various concentrations between 10 and 140 μg/mL, however not at the highest concentration (150 μg/mL). Isolate 7920 contained a $CdCl_2$ resistant gene, however it was only able to grow to 10 μg/mL. There were also nine isolates which had no *cadA* genes but were able to grow at 10 μg/mL.

3.3. Antimicrobial susceptibility

Five antibiotics used for the treatment of listeriosis were tested against the *L. monocytogenes* isolates (Fig. 1). All the *L. monocytogenes* isolates displayed sensitivity to the antibiotics tested in this study (amoxicillin, gentamicin, penicillin, ciprofloxacin and trimethoprim/sulfamethoxazole).

3.4. Food production stress determinants

All isolates were assessed for the presence of genetic determinants relating to various stress conditions experienced within the food production environment, used as a mitigation strategy to reduce growth and/or survival of *L. monocytogenes*. These stress conditions included thermal treatment, low temperatures, acidification, oxidation, osmotic stress, the use of bacteriocins or nisin, and high hydrostatic pressure (HHP). A screening database of genetic determinants associated with each condition identified within the literature was selected to determine the potential of strains to survive within the FPE. The genetic determinants selected for heat, acid, cold, osmotic, bacteriocin/nisin and HHP stresses were present in all isolates (Supplementary Table 3). The stress survival islets (SSI) were also assessed, with SSI-1 present in 34 of the 52 isolates (65.4%) from ST3, 7, 8, 9, 12, 155, 204 and 321 (Fig. 1). The five isolates from ST121 were positive for SSI-2 (9.6%), and an SSI genotype harbouring an *LMOf2365_0481* gene homolog was present in 13 of the 52 isolates (25.0%) from ST1, 2 and 101.

3.5. Cadmium and disinfectant genes

The *L. monocytogenes* isolates were analysed for the presence of cadmium genes *cadA1C-A6C* (Fig. 1). There were 19 isolates which had no *cadA* genes present; the *cadA1* gene was present in 20 isolates, *cadA2* was represented in five isolates, four isolates had *cadA4* and five isolates had *cadA5* present. No isolates had the *cadA3* or *cadA6* gene. Isolate 7929 had two *cadA* genes present, *cadA2* and *cadA4*. Two *cadA* genes were also present in isolate 7533, *cadA2* and interestingly, it also contained a transposon with a novel *L. monocytogenes* cadmium resistance gene, referred to here as *cadA7*. A nucleotide BLAST search of NCBI nucleotide database identified four other *L. monocytogenes* strains also contain this transposon and the novel *cadA7* gene homolog. This

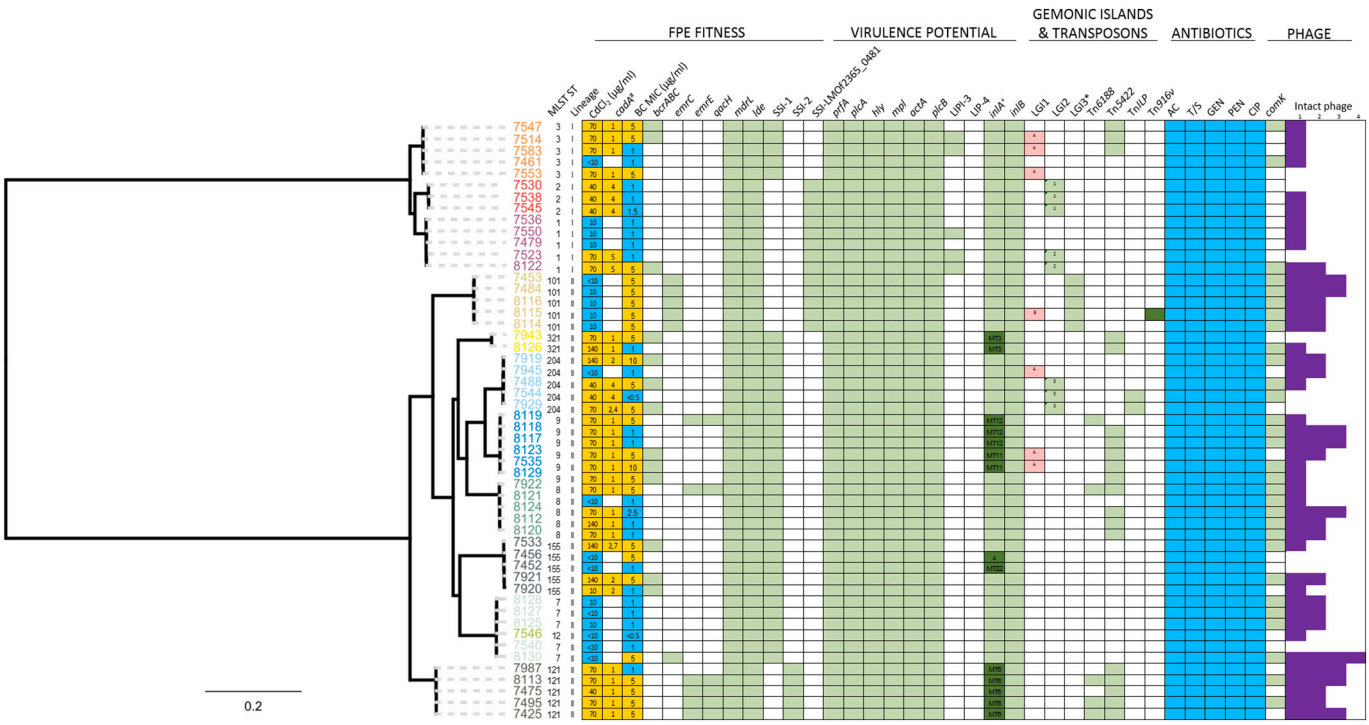

Fig. 1. Phylogeny and genetic determinants of 52 *L. monocytogenes* isolates relating to survival within the FPE, virulent potential and therapeutic treatment potential. Character designations are as follows: #, numbers designated *cadA* gene type; ^, pLi0048 – elements of the pLi100 are present however we were unable to close or identify full plasmid. This plasmid is known to carry cadmium resistant genes; +, *inlA* PMSC type; Δ, 70 AA deletion; A, phage insert in the *lmo1703-lmo1702* region; B, transposon insert in the *lmo1703-lmo1702* region (*Tn916* variant); 1, LGI2 insert within the *EGD-e LMO2257* gene; 2, LGI2 variant; 3, LGI2 insert in the *yfbR* gene; *, LGI3 lacking the *cadA1C* cassette; yellow, resistant phenotype; light green, gene is present; dark green, gene is present – does not match wildtype; light red, LGI1 is absent however there is alternative genes present within the *lmo1703-lmo1702* region; blue, sensitive phenotype; orange, *comK* phage is present; purple, number of intact phage regions present. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

transposon was also identified in Enterobacteriaceae strains suggesting direct or indirect horizontal gene transfer occurring between *Enterococcus* and *Listeria* (Fig. 2). No other isolates within this study contained the *cadA7* gene. Amino acid sequence identity of the published cadmium genes and the novel *cadA7* gene was determined, with *cadA7* sharing the highest amino acid percentage identity with *cadA2*, 75.74% (Supplementary Fig. 1); however, when compared to *E. faecalis* ATCC 29212 genome the novel *cadA7* displayed 100% coverage and 99.95% nucleotide identity.

A variety of genes and mechanisms providing resistance to disinfectants were assessed against the isolates in this study (Fig. 1). All isolates were found to contain the *mdrL* and *lde* efflux pumps. The *bcrABC* cassette was present in 13 isolates covering ST1, 3, 9, 155, 204 and 321. Six isolates in total were positive for *ermB* and *qacH* from ST8, 9 and 121. The *emrC* gene was present in six isolates, one isolate from ST7 and all the isolates from ST101. The ST101 isolates were the only whole ST group in which all contained the same disinfectant resistance gene.

3.6. Plasmids, prophages and transposons

Plasmid replicons were identified in 26 of the 52 isolates with PlasmidFinder. The identified regions were further interrogated in the draft genomes. A total of 13 closed plasmids and 12 draft open plasmids were identified (Table 1). Plasmids were present in ST3, ST8, ST9, ST121, ST155, ST204 and ST321 isolates (Fig. 3). ST121 was the only group in which plasmids were found in all five isolates and displayed a 95.9% pairwise identity with pLM6179, however only three of these plasmids were closed following sequence analysis. Genes shared across the plasmids, and not restricted to a single ST, included heavy metal and disinfectant resistance genes including the *bcrABC* operon *cadAC* operon, and genes for copper, zinc and arsenic resistance; stress response genes including UV damage repair protein, oxidative and heat stress

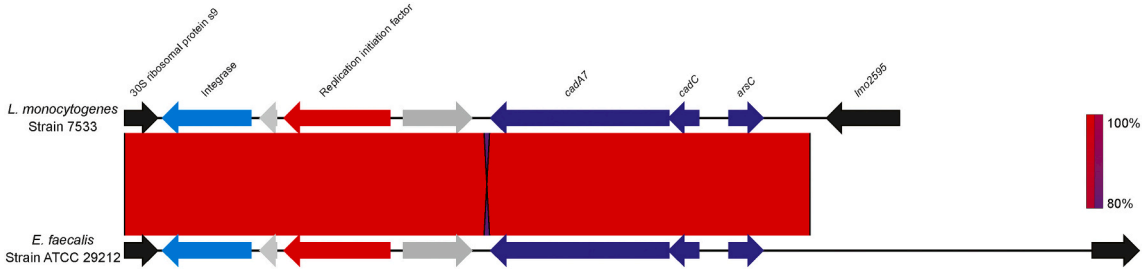

Fig. 2. The novel *cadA7* gene. Transposon identified in isolate 7533 inserted between 30S ribosomal protein S9 and *lmo2595*, compared to *E. faecalis* ATCC 29212. Integrase genes are in light blue, replication genes are in red, heavy metal resistance genes are in purple. Hypothetical genes or those with an unknown function are shaded grey. Nucleotide sequence identity of transposon where shared, ranged from 80 to 100% as depicted by the percentage homology bar. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

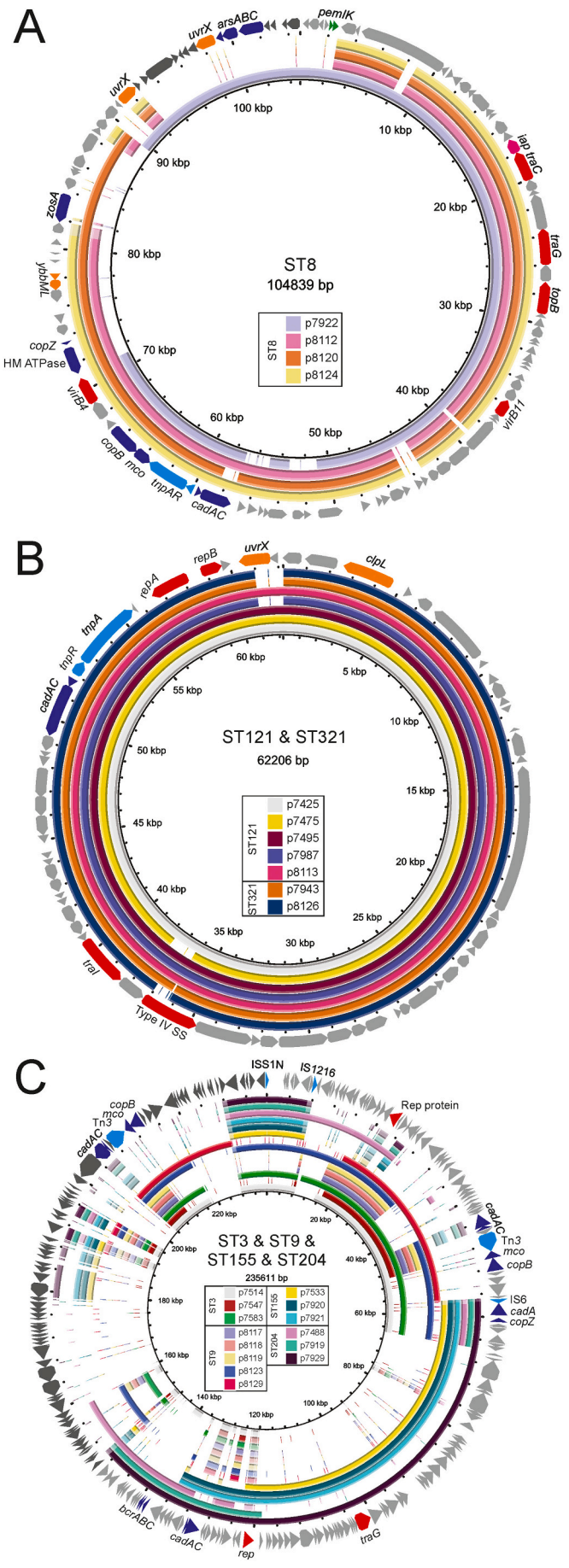

Fig. 3. BRIG comparison of plasmids identified within 26 isolates within this study. Each ring represents the plasmids of the individual isolates. The plasmid pangenome reference contains all the unique genetic features of the closest related plasmid identified in NCBI and segments of plasmids from the respective pST groups from this study which are not represented in NCBI, combined into a single contig reference (outside ring). (A) pST8 utilises the genome of pLM1686 (MK134858) (light grey) and p7922 (dark grey) as reference pangenome. (B) pST121 and pST321 utilises the p6179 as reference genome. (C) pST3, pST9, pST155 and pST204 utilises the genome of pN1-011A (light grey) and pR479a (dark grey) as reference pangenomes. Annotated genes are colour coded to represent genetic markers as follows: red – replication, light blue – transposases, dark blue – heavy metals, orange – stress response, pink – invasion associated, green – toxin/antitoxin and grey – hypothetical proteins. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

response genes; invasion related genes; toxin/anti-toxin genes; genes involved in DNA replication, translation, recombination and conjugation; transposon genes; however most genes were hypothetical proteins.

Phaster identified 52 intact phage regions across 43 isolates. Nine isolates had no phage regions, most isolates had a single phage region, two regions were identified in 15 isolates and three and four regions identified in four and one isolate respectively. An additional analysis of the *comK* phage insertion site identified 21 isolates with a full length *comK* gene and 31 isolates with a *comK* prophage disruption (Fig. 1). Transposon elements were identified in 27 of the 52 isolates. Transposon Tn*6188* was present in five isolates, Tn*5422* was identified in 20 isolates, Tn*ILP* was present in two isolates and Tn*yfbR* was identified in six strains. Seven isolates contained two transposons.

3.7. Virulence determinants and genomic islands

The *Listeria* pathogenicity and genomic islands were assessed against the panel of isolates (Fig. 1). The LIPI-1 virulence cluster was present in all isolates, along with the *inlB* gene. The LIPI-3 element was found in three isolates (7523, 7550 and 8122) from ST1 and two isolates (7514 and 7583) from ST3. No isolates harboured LIPI-4; this island has only been identified in CC4 isolates, which were not included in this study. The LGI2 was present in eight isolates, in either one of two insertion locations; within the *LMOSA2140* (homolog of *LMOf2365_2257*) gene originally identified in the strain ScottA (Lee et al., 2013), or within the *yfbR* gene (Fox et al., 2016). The three isolates with the *LMOSA2140* LGI2 insertion region were from ST2 and three ST204 strains contained the *yfbR* LGI2 insert. Interestingly, an LGI2 variant was also identified from two ST1 isolates within a transmembrane protein that displays distant homology to *ydbT* gene. This LGI2 variant harbours an additional gene, a *metC* homolog, within the LGI2 region (Fig. 4). The recently reported *Listeria* genomic island 3 was partially identified in the five ST101 isolates only; however, they were missing the 6248 bp region containing the *cadAC* homolog, recombinase and Tn*3* family transposase.

3.8. Internalin A (inlA) analysis

The *inlA* gene was assessed for the presence of mutations resulting in premature stop codons (PMSCs), truncation or a full length *inlA* gene (Fig. 1 and Supplementary Fig. 2). Thirty-eight of the isolates contained a full length *inlA* gene. Thirteen isolates contained PMSCs. All ST121 isolates (7425, 7475, 7495, 7987 and 8113) contained mutation type 6 at AA 492, as previously described (Van Stelten et al., 2010). Isolates 8123 and 7535 contained a PMSC at AA 685, resulting in mutation type 11. Both these isolates are ST9, from which this mutation type has been previously associated with (Van Stelten et al., 2010). Mutation type 12, the result of a PMSC at AA 576 was identified in three isolates from ST9 (8117, 8118 and 8119) and mutation type 3, the result of a PMSC at AA 700, was identified in isolates 8126 and 7943 from ST321. A novel

(caption on next column)

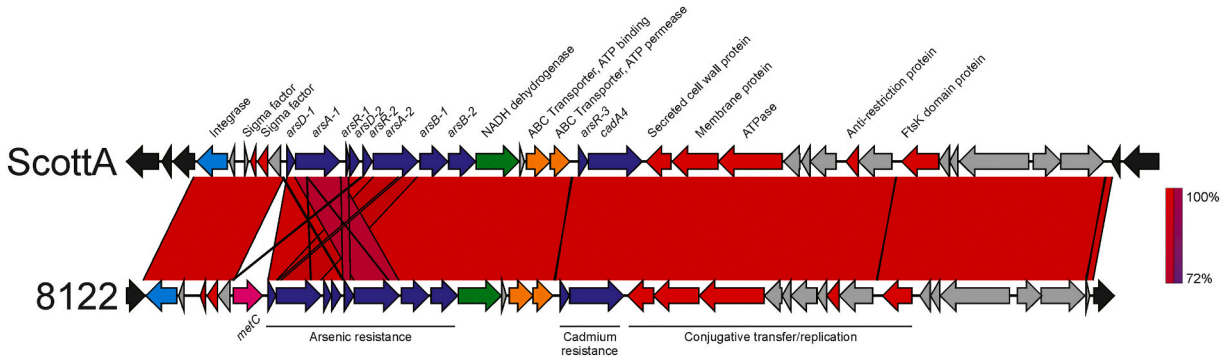

Fig. 4. LGI2 variant identified in isolate 8122. A variant of LGI2 inserted in a transmembrane protein within isolate 8122 and 7523. Annotated genes are colour coded to represent genetic markers as follows: black – flanking genes, light blue – integrase, dark blue – heavy metal and antimicrobial resistance, orange – metabolism and transport, red - transposon system and regulatory genes, pink - virulence, green – stress resistance, grey – hypothetical proteins. Sequence identity where shared, ranged from 72 to 100% as determined by the percentage homology bar. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

PMSC was identified in isolate 7452, the result of a frame shift mutation from an AG insertion at nucleotide position 183 producing an *inlA* gene of 67 AA in length. Isolate 7456 contained an in-frame 70 AA deletion within the B-repeat region (B- repeat region 2 and 3).

3.9. *Listeria genomic island 1 analysis*

None of the isolates harboured the *Listeria* genomic island 1 (Fig. 1). To confirm this, we manually inspected the hypervariable region between *lmo1703-lmo1702* locus, an RNA methyltransferase gene and the fosfomycin resistance gene *fosX* respectively, for inserts in all isolates. A phage (ϕRNA-MT) insert was present in six of the isolates (7514, 7535, 7553, 7583, 7945 and 8123) and a novel insert was harboured in isolate 8115. This novel insert was not present in any of the other isolates. A BLAST search resulted in a 64% query coverage and 90.73% nucleotide identity with Tn*916* from *Bacillus subtilis* (which has over 98% identity with Tn*6198* of *L. monocytogenes* TTH-2007) and a 64% query coverage and 91% identity with an integrated chromosomal element ICESpnIC1 identified in *St. pneumoniae* isolate 9611+04103 (Fig. 5). This Tn*916* variant insert between *lmo1703* and *lmo1702* in isolate 8115 is approximately 23,275 bp in length, has a GC content of 37.5% and contains a Clp protease ATP-binding subunit *clpA*, as well as coding sequences for FtsK/SpoIIIE family protein, a Tn*916* transcriptional regulator, an anti-restriction protein, lipoprotein, a *XRE* family transcriptional regulator, an efflux *ABC* transporter, and a site-specific recombinase with the remaining genes being hypothetical proteins.

3.10. *Antimicrobial resistance determinants*

The *L. monocytogenes* isolates were analysed for the presence or absence of a variety of antimicrobial genes associated with conferring resistance to frequently used antibiotics in the treatment of listeriosis or other diseases (Supplementary Table 4). Resistance genes from the following selected antibiotics classes: trimethoprim, tetracycline except

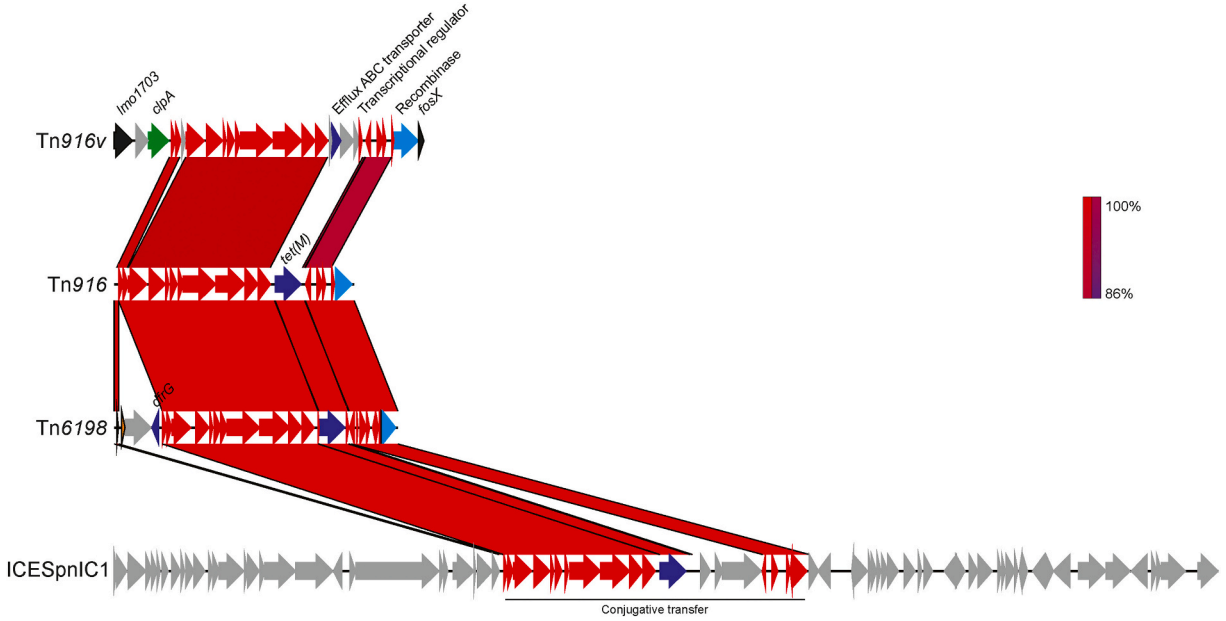

Fig. 5. Novel insert identified in isolate 8115. The insert was identified in the hypervariable region of isolate 8115 between genes *lmo1703-lmo1702*, compared to Tn916, Tn6198 and *S. pneumoniae* 9611+04103 ICESpnIC1. Sequence identity where shared, ranged from 78 to 100% as determined by the percentage homology bar. EGD-e flanking genes, *lmo1703* and *lmo1702* are shown in black; red genes are transposon systems and regulatory genes, light blue – integrase, dark blue – heavy metal/antimicrobial resistance, green – stress response and grey – hypothetical proteins. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

for the *tetA*-like resistance protein, vancomycin, erythomycin, amoxicillin and aminoglyosides along with the penicillin *mecC* protein were not detected in any isolates. The AMR genes selected relating to flurquinolones (second generation), penicillin (except *mecC*), sulfonamide, fosfomycin, lincomycin, fusidine, quinolone and cephalosporins were present within all isolates. There were no AMR genes or class specific to an isolate, lineage or ST. Potential resistance mechanism beyond known AMR genes were not examined.

4. Discussion

This study utilised phenotypic and genotypic analyses of whole genome sequences to assess the potential of *L. monocytogenes* to survive within the FPE, cause disease and provide insights into antimicrobial resistance relevant to control in the FPE, or to treatment of human infection.

4.1. Virulence potential

Clonal complex 1 (CC1) and 2 (CC2) are well established as being associated with clinical infections (Maury et al., 2016; Yin et al., 2019), and therefore the presence of genomic regions relating to hypervirulence in food isolates is of interest. In this study ST1 and ST2 isolates did not contain the SSI-1 or SSI-2 islands, however, the SSI-alternative, *LMOf2365_0481* homolog, was present in all isolates. A study by Harter et al. (2017) reported the presence of this SSI-alternative to be common among clinical strains, however its function at this stage is undetermined. While CC1 and CC2 did not contain SSI-1 or SSI-2, the isolates did contain all but one of the stress resistance-associated genes screened, with a role in heat, cold, acid, osmotic, oxidative or nisin stress response, suggesting that they are capable of surviving within the FPE, or in food. Horlbog et al. (2018) found CC1 strains were able to recover more quickly after salt stress, suggesting these strains could be able to proliferate faster within food environments. All isolates of CC1 and CC2 contained full length *inlA* genes; three of the CC1 isolates contained LIPI-3, suggestive of increased virulence potential.

Within Australia, CC3 and CC204 are widely distributed (Jennison et al., 2017), with these strains analysed in this study all containing LIPI-1, full length *inlA*, SSI-1, and all the various processing related stress genes. Neither CC3 nor CC204 contained the LGI1, however, three of the CC3 strains and one of the CC204 strains contained a phage insert in the LGI1 hypervariable position between *lmo1703* and *lmo1702*. Interestingly, two of the CC3 isolates contained the phage insert instead of LGI1. Increased bacterial colonisation and hypervirulence has been reported in isolates which encode a full length *inlA*, LIPI-1 and LIPI-3 (Maury et al., 2016; Yin et al., 2019), suggesting the isolates in this study which contain these have increased virulence potential, and with the addition of SSI-1 may also have increased FPE fitness. The CC204 strains which were negative for LGI1 and the ϕRNA-MT phage insert, contained LGI2. *Listeria* genomic island 2 contains cadmium and arsenic resistance genes, providing increased environmental survival potential; interestingly, also in both this study and Lee et al. (2017), LGI2 was common in CC2 strains, which are also prevalent in human cases. Although LGI2 requires further characterisation, it is prevalent in clonal complexes linked to hypervirulence and has been suggested to contribute to virulence (Lee et al., 2017).

Premature stop codons and mutations in the *inlA* gene resulting in secretion of *inlA* instead of being attached to the bacterial cell wall have been associated with reduced invasion and virulence ability (Ferreira da Silva et al., 2017; Gelbíčová et al., 2015; Van Stelten et al., 2016). In this study, 13 isolates were identified to carry one of five types of mutations, suggesting these isolates may have a reduced virulence potential. We identified a novel PMSC at 67 AA resulting from an AG insertion at position 183 bp producing a frameshift, referred to as mutation type 22. This mutation occurs within the signal cap region of the *inlA* protein. Mutation types 4 and 15 occurring at AA positions 9 and 77 respectively have been shown to affect invasion ability (Van Stelten et al., 2010), indicating mutation type 22 has the potential to also have reduced invasiveness, however, in vitro cell invasion assays and/or mouse model virulence assays will be required to confirm this theory. In addition, a 70 AA deletion within the B-repeat region was identified in isolate 7456. Deletion of the B-repeat region between AA 517 and 707 by Lecuit et al. (1997) resulted in similar invasiveness level to those with the WT *EGD-e inlA* protein, suggesting this deletion within the B-repeat region does not contribute to a strains ability to invade cells. All ST121 isolates contained the type 6 mutation and ST321 isolates contained mutation type 3. In addition, all but two *inlA* mutant isolates had cadmium resistance, contained either SSI-1 or SSI-2, and harboured plasmids. All of these isolates harboured LIPI-1. The majority of *inlA* mutants are commonly associated with the FPE and food isolates (Nightingale et al., 2005; Van Stelten et al., 2016), therefore the presence of these genes in the *inlA* mutant isolates are suggestive of increased survival within the FPE.

4.2. FPE survival potential

Agricultural practices and industrial pollution have resulted in increased levels of various heavy metals in the environment, and as such bacteria require resistance determinants in order to tolerate these substances, particularly heavy metals which are not required for cellular processes. Cadmium resistant determinants are widely distributed and are commonly associated with *L. monocytogenes* strains repeatedly isolated from food sources (Parsons et al., 2017). In this study, 32 isolates were capable of growing at levels above 40 μg/mL $CdCl_2$, which was associated with the presence of at least one cadmium resistant determinant, with similar results observed in previous studies (Haubert et al., 2019; Lee et al., 2013; Mullapudi et al., 2010; Ratani et al., 2012; Xu et al., 2019). Interestingly, in this study we did not identify the *cadA3* resistant determinant which is present as an integrating chromosomal element in a variable genomic region, that in other strains may contain diverse cassettes like LIPI-3 (Parsons et al., 2019).

The novel *cadA7*, identified in this study, contained all three key motifs, DKTGT, CPC and CTNCA, characteristic of the *cadA* protein family (Bal et al., 2003; Parsons et al., 2017). Parsons et al. (2017) identified an amino substitution in the CTNCA → CANCA motif in *cadA4* suggesting this substitution most likely, in conjunction with other elements, influencing *cadA4*'s reduced tolerance of 35 μg/mL to cadmium. In this study, the three key motifs of the novel *cadA7* matched the *cadA1-cadA3* sequences, which are purportedly associated with cadmium resistance of 140 μg/mL or higher, potentially suggesting *cadA7* may confer similar levels of resistance (Parsons et al., 2017). The single isolate harbouring *cadA7* in this study also contained *cadA2*; as such, further research is required to establish the resistance level conferred by *cadA7*, and to determine if it has a potential role in virulence.

The presence of disinfectant resistant genes in this study was associated with resistance to BC (≥5 μg/mL) in all but two isolates, with 50% of the isolates displaying a MIC of 5 μg/mL or higher. The capability of isolates to grow at higher levels of disinfectants like BC is being increasingly reported (Mullapudi et al., 2008; Møretrø et al., 2017). In addition, the *L. monocytogenes* strains' tolerance to disinfectants has been correlated with cadmium resistance and increased survival within the FPE being associated with subinhibitory levels of disinfectants (Martinez-Suarez et al., 2016; Mullapudi et al., 2008; Ortiz et al., 2014). Therefore, an evaluation of the level of resistance of cadmium and BC is important to understand the survival potential *L. monocytogenes* may have in the FPE.

Genomic islands have the potential to contain genes to improve the fitness of an isolate, while also being implicated in potential horizontal gene transfer (Palma et al., 2020); therefore, the presence of these islands might lead to increased FPE survival or pathogenic potential. Of the genomic islands identified in *L. monocytogenes*, LGI1 and LGI3 have been associated with survival and persistence in the FPE (Kovacevic et al., 2016; Palma et al., 2020), with LGI2 potentially providing

increase survival and persistence within the FPE as well as virulence potential (Lee et al., 2013; Lee et al., 2017).

In this study, LGI2 was the only full-length island present in six isolates, inserted within one of two genes, *LMOSA2140* or *yfbR*. This has the potential to provide increased virulence and environmental fitness. In addition, an LGI2 variant was identified in two ST1 isolates within a transmembrane protein that displays distant homology to the *ydbT* gene from *Bacillus*. The *ydbT* in *Bacillus subtilis* strains has been reported to provide resistance to bacteriocins produced by *B. amyloliquefaciens*, an important function particularly in natural environmental reservoirs like soil (Butcher and Helmann, 2006). The LGI2 variant shows high homology with LGI2 and maintains the arsenic and cadmium resistant determinants along with various metabolism, transport, stress resistance, transposon and regulatory genes. However, the LGI2 variant contains an additional cystathionine β-lyase (*metC*) gene. A previous study demonstrated that disruption of *metC* in *Salmonella*, reduced strain virulence in a mouse model (Ejim et al., 2004). This suggests the LGI2 variant may play a role in virulence as well as survival within food and the food environment; however, this needs to be further confirmed experimentally.

In this study, the LGI3 variant which lacks the *cadA1C* cassette was identified in all ST (CC) 101 isolates. The LGI3 element was first identified in CC101 isolates by Palma et al. (2020) and found to harbour a *cadA1C* cassette; however, a search of the NCBI genome database identified a smaller LGI3 variant lacking the *cadA1C* cassette in the *L. monocytogenes* strain ATCC 51775 (ST222). In comparison, the CC101 isolates from this study also contained the LGI3 variant, suggesting CC101 strains may display either LGI3 genotype.

Instead of LGI1, six isolates contained a phage insert and one isolate contained a Tn*916* variant insert, in the associated insertion locus. This Tn*916* variant shares similarity with Tn*916* and Tn*6198*; however, it lacks the tetracycline (*tetM*) and the trimethoprim (*dfrG*) resistant genes. Interestingly, an efflux ABC transporter is present on the Tn*916* variant, which shares homology to efflux systems. Further experimental work is required to determine its function in this transposon, and the ability of this transposon to transfer to other *Listeria* strains, or other bacterial species.

4.3. Therapeutic treatment potential

Traditionally, listeriosis is treated with a β-lactam (penicillin, ampicillin or amoxicillin) either alone or in combination with an aminoglycoside, typically gentamicin (Grayo et al., 2008; Knudsen et al., 2013; Olaimat et al., 2018; Temple and Nahata, 2000) or trimethoprim and sulfamethoxazole combination for patients with a β-lactam sensitivity(Bertrand et al., 2016; Wilson et al., 2018). While in this study all the isolates were sensitive to the five clinically relevant antibiotics tested, there has been reports in the literature of resistance to gentamicin, penicillin, ciprofloxacin and trimethoprim/sulfamethoxazole along with a variety of other antibiotics and importantly reports of resistance to multiple antibiotic classes (Arslan and Özdemir, 2020; Kuan et al., 2017; Obaidat et al., 2015; Obaidat and Stringer, 2019; Welekidan et al., 2019). Our study provides a timely contribution to the current state of AMR in *L. monocytogenes* and does not highlight any resistance concern among food isolates in this study.

In this study, we identified a novel cadmium gene, *cadA7* as part of a transposon insert, a variant of LGI2, as well as a novel insert in the hypervariable region LGI1, in the latter sharing similarity to a Tn*916* transposon. The identification of these novel genes and inserts contributes to our understanding of the *L. monocytogenes* pangenome, in particular to elements relating to survival ability and pathogenic potential. The isolates analysed in this study showed potential to survive and persist within the FPE, with all isolates containing one of the SSIs, various genes relating to stressors present in the FPE to reduce bacteria, in addition to a high portion of strains containing cadmium or disinfectant resistance genes. Hypervirulent strains of *L. monocytogenes* have been previously reported, with some isolates from CC1 and CC3 in this study harbouring genes associated with this virulence status, suggesting a concern to public health.

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CRediT authorship contribution statement

JG, PSC and EF conceived and designed the study. JG performed the experiments. Data was analysed by JG, EF, PSC, JPB, MK and CK. JG and EF drafted the manuscript. All authors corrected and approved the manuscript.

Declaration of competing interest

None.

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REVIEW
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Novel Biocontrol Methods for *Listeria monocytogenes* Biofilms in Food Production Facilities

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High mortality and hospitalization rates have seen *Listeria monocytogenes* as a foodborne pathogen of public health importance for many years and of particular concern for high-risk population groups. Food manufactures face an ongoing challenge in preventing the entry of *L. monocytogenes* into food production environments (FPEs) due to its ubiquitous nature. In addition to this, the capacity of *L. monocytogenes* strains to colonize FPEs can lead to repeated identification of *L. monocytogenes* in FPE surveillance. The contamination of food products requiring product recall presents large economic burden to industry and is further exacerbated by damage to the brand. Poor equipment design, facility layout, and worn or damaged equipment can result in *Listeria* hotspots and biofilms where traditional cleaning and disinfecting procedures may be inadequate. Novel biocontrol methods may offer FPEs effective means to help improve control of *L. monocytogenes* and decrease cross contamination of food. Bacteriophages have been used as a medical treatment for many years for their ability to infect and lyse specific bacteria. Endolysins, the hydrolytic enzymes of bacteriophages responsible for breaking the cell wall of Gram-positive bacteria, are being explored as a biocontrol method for food preservation and in nanotechnology and medical applications. Antibacterial proteins known as bacteriocins have been used as alternatives to antibiotics for biopreservation and food product shelf life extension. Essential oils are natural antimicrobials formed by plants and have been used as food additives and preservatives for many years and more recently as a method to prevent food spoilage by microorganisms. Competitive exclusion occurs naturally among bacteria in the environment. However, intentionally selecting and applying bacteria to effect competitive exclusion of food borne pathogens has potential as a biocontrol application. This review discusses these novel biocontrol methods and their use in food safety and prevention of spoilage, and examines their potential to control *L. monocytogenes* within biofilms in food production facilities.

Keywords: *Listeria monocytogenes*, biofilms, biocontrol, bacteriophages, bacteriocins, endolysins, competitive exclusion, essential oils

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INTRODUCTION

Listeria monocytogenes is a Gram-positive, rod shaped, facultative anaerobe capable of causing food borne illnesses particularly in high-risk population groups including the elderly, immune compromised, pregnant women, and neonates (Farber and Peterkin, 1991). While *L. monocytogenes* associated illness is not as common as that of other food borne pathogens like *Salmonella, Campylobacter*, or *Escherichia coli*, its mortality rate can be considered the highest. Approximately 30 % of invasive listeriosis cases lead to mortalities with most requiring hospitalization, and therefore demanding *L. monocytogenes* can be considered as a food borne pathogen of public health importance (Lomonaco et al., 2015; Véghová et al., 2016). Due to its ubiquitous nature, *L. monocytogenes* poses a food safety risk as it is frequently introduced into the processing environment through raw ingredients. *L. monocytogenes* can adhere to a variety of abiotic surfaces with some strains persisting for numerous years and acting as a source of continuous cross contamination (Fox E. et al., 2011; Coughlan et al., 2016; Colagiorgi et al., 2017).

Due to significant food safety risks, the control of *L. monocytogenes* has become a regulatory requirement that food business operators must adhere to. Regular cleaning, disinfecting, and sanitizing of food contact and non-food contact surfaces are required as part of a sanitation plan that also incorporates maintenance of equipment and buildings, pest control, and general hygiene. In addition, the implementation of good manufacturing practices and effective hazard analysis critical control point plan aids in reducing the risk of food borne illness (Drew and Clydesdale, 2015). However, *L. monocytogenes* is a difficult organism to eradicate and its presence still occurs even with the best management plans (Tompkin, 2002; Drew and Clydesdale, 2015).

While the exact mechanisms can be unclear for how *L. monocytogenes* is able to persist in food production environments (FPEs) so successfully, researchers have proposed that there are numerous factors at play. Poorly maintained equipment, surfaces, and unhygienic factory design can result in niches containing adequate nutrients, water, and protection from cleaning allowing bacteria to survive and grow while also introducing bacteria to subinhibitory levels of disinfectants (Carpentier and Cerf, 2011; Fox E.M. et al., 2011; Ibba et al., 2013; Møretrø et al., 2017). Typically disinfectants, when applied correctly, can sufficiently inhibit the colonization of introduced planktonic cells; however, dosing failures and applying disinfectants to wet surfaces can result in equipment being inadequately disinfected and bacteria being exposed to subinhibitory chemical levels (Martinez-Suarez et al., 2016; Møretrø et al., 2017). Incorporating desiccation processes has been shown to increase the effectiveness of disinfections procedures (Overney et al., 2017); however, an ample amount of drying time is difficult when continuous or even daily production runs are required. It is also important to note the difference between resistance, an increase in concentration or time required to exert the same reduction, and tolerance, an adaptation in a microbe's susceptibility potentially the result of exposure to subinhibitory levels (Cerf et al., 2010; Ortega Morente et al., 2013). For example, some *L. monocytogenes* strains are known to carry genes for disinfectant chemical efflux pumps, such as *qacH* and *bcrABC*. The distribution of these genes tends to vary on a strain by strain basis instead of being unique to a specific lineage or subtype (Dutta et al., 2013; Ortiz et al., 2015; Møretrø et al., 2017). Although it has been reported that these genes only result in tolerance to quaternary ammonium compounds at levels far below the concentrations actually used in the food industry (Tezel and Pavlostathis, 2015), the ability to form biofilms is also a crucial factor in the survival of *L. monocytogenes*. Biofilms are composed of numerous cells attached to each other or an abiotic surface surrounded by an extracellular matrix containing a mixture of polysaccharides, proteins, and extracellular DNA (da Silva Fernandes et al., 2015; Fagerlund et al., 2017). This extracellular matrix provides a protective barrier around the internalized microbial cells from desiccation and heat, contributes to increased adhesion, and is a reservoir of nutrients (Colagiorgi et al., 2016). In addition, biofilms can impede the activity of antimicrobial agents as the matrix limits their diffusion potential and contains cells with differing susceptibility while also allowing for the acquisition of new genetic traits like those mentioned above through horizontal gene transfer. Further, biofilms typically consist of multiple species that can allow for the colonization of transient strains or provide increased attachment and survival to strains not typically good biofilm formers (Coughlan et al., 2016).

THE BIOCONTROL METHODS MOVEMENT

While tolerance to disinfectants and sanitizers is not considered as significant an issue as antibiotic resistance, their continued use and potential ineffectiveness against biofilms warrant new strategies for the control of *L. monocytogenes*. As consumers become more conscious of food safety significance, the use of novel biocontrol methods is gaining further interest. This return to biocontrol methods of microbes and plants has the potential to relieve some of the tolerance to disinfectants and decrease some of the selective pressures that their overuse has on maintaining resistance markers (Coughlan et al., 2016). Biocontrol methods with potential to act against listerial biofilms include bacteriophages, their endolysins, competitive bacterial species and their antimicrobial products, bacteriocins, and plant-derived products and will be discussed in this review.

BACTERIOPHAGES

The most abundant microorganism on earth, bacteriophages (phages) are viruses that infect bacteria for propagation, live naturally in the environment, and anywhere host bacteria are found (Bai et al., 2016; Pérez Pulido et al., 2016). Phages are classified based upon their morphology (head and

tail, either contractile or non-contractile, or no tail), nucleic acid (double stranded or single stranded; deoxyribonucleic or ribonucleic acid), and life cycle, which is of most relevance for biocontrol. There are two types of life cycles phages can undergo after entering the bacterial cell: the lysogenic cycle (temperate phages) or the lytic cycle (**Figure 1**). Phages may be capable of a lysogenic cycle that converts to the lytic cycle in unfavorable conditions, or undergo a solely lytic life cycle. Temperate phages are not suitable as a biocontrol agent as integration into the host genome may result in increased pathogenicity through horizontal gene transfer (Hagens and Loessner, 2007; Salmond and Fineran, 2015). In comparison, lytic phages are ideal as a biocontrol agent due to their fast-lytic action.

Although identified over a hundred years ago, interest in phages has only recently been reignited with the rise of antibiotic resistance among bacteria (Hagens and Loessner, 2007). The utility of phages has included the treatment of diseases in humans and animals, typing of bacterial strains, decontaminating meat carcasses after slaughter, and targeted inactivation of pathogenic and spoilage bacteria on food contact and non-contact surfaces as well as surfaces of ready to eat products and during packaging and storage (Hagens and Loessner, 2007; Strauch et al., 2007). The application of phages as an innovative approach to control biofilms in the FPE is also beginning to be explored. While there has been great achievement in the use of phages from a therapeutic perspective, their success in the FPE is not as simple. Factors like the composition and structure of the biofilm, temperature, the metabolic state of the bacteria in the biofilm, the extracellular matrix in general, food components, and nutrients all provide additional challenges to the effectiveness of phage application (Parasion et al., 2014). While there have been some reports of phage resistance (Fister et al., 2016), it occurs more gradually than the development of antibiotic resistance as phages are able to mutate continuously, like bacteria, and resistance is further slowed by using a combination of phages active against the one bacterial species (Hagens and Loessner, 2007; Sadekuzzaman et al., 2017). There is a substantial amount of research conducted on phages' ability to protect food from *Listeria*, with two commercial *Listeria* phage products, ListShield™ and Listex™ P100 approved as food preservatives with the generally recognized as safe status since 2006. However, studies investigating the efficacy of these products and other *Listeria* phages against biofilms are few, with most having focused on Listex™ P100.

Biofilm maturity has the potential to reduce the efficacy of phage treatment, as well as any control method. Various

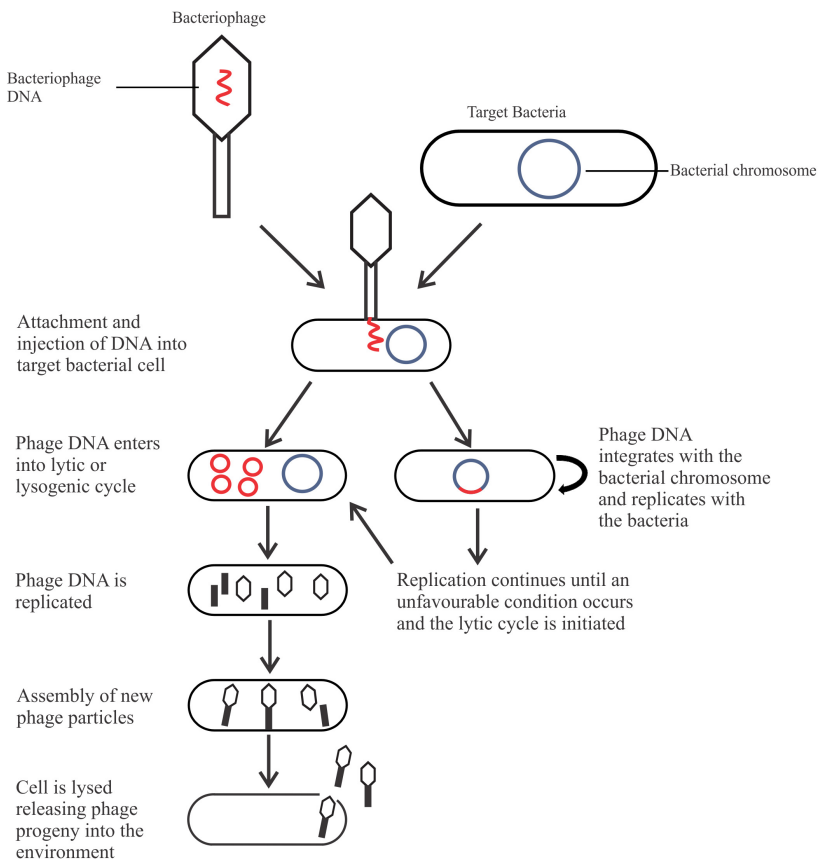

FIGURE 1 | The life cycles of a bacteriophage.

studies have examined this concept utilizing preformed biofilms at various maturity levels, ranging from 24 h to 2 weeks, with most studies reporting a minimum 1-log reduction. Most studies to date have utilized stainless steel as the surface to form *L. monocytogenes* biofilms and examine the efficacy of bacteriophage treatments. This reflects the widespread presence of these surfaces, both food contact and non-contact in food processing environments. The success of bacteriophage treatments at inactivating *L. monocytogenes* biofilms on these surfaces, however, has shown mixed results. A number of studies demonstrated promising results when Listex^{TM} P100 was applied to *L. monocytogenes* biofilms on stainless steel, with reductions in the order of 5-log being achieved (Soni and Nannapaneni, 2010; Montañez-Izquierdo et al., 2012). Both of these studies used an application treatment of 24 h at ambient room temperature. Iacumin et al. (2016) also applied Listex^{TM} P100 for 24 h at 20°C onto stainless steel wafers and report the complete elimination of *L. monocytogenes* biofilm. This prolonged treatment application, however, in many cases is not practical in an FPE. In addition, Iacumin et al. (2016) pressed the stainless steel wafer onto an agar plate to replicate the process of cross-contamination in the FPE; however, it did not take into consideration the phage products ability or inability to act on biofilms in the crevices or corners where these might be thicker than a flat surface.

A shorter treatment time of 2 h was applied by Sadekuzzaman et al. (2017) when running a similar inactivation test with ListShield^{TM}; however, this was associated with a much lower inactivation of just 2-log when applied to *L. monocytogenes* biofilm on stainless steel. This was even less effective on a rubber surface, achieving a 1-log reduction in *L. monocytogenes* cell numbers. The results of Sadekuzzaman et al. (2017) also reflect those observed by Gutiérrez et al. (2017) who saw a similarly low inactivation achieved by a 4 h ListShield^{TM} treatment, typically 1-log or less. Although the latter study did show greater inactivation with Listex^{TM} P100 under the same treatment conditions, the Listex^{TM} P100 commercial phage preparation showed a reduced activity range, only capable of infecting 7 of the 11 strains tested. An important aspect in phage application is the ratio of phage to bacteria known as the multiplicity of infectivity. To increase the likelihood the phage will infect the bacterium, the phage needs to be at a higher ratio than the number of target bacterial cells (Montañez-Izquierdo et al., 2012). High multiplicity of infectivity has been reported to result in efficient phage treatment with one study recommending a multiplicity of infectivity around five was required for adequate reductions by Listex^{TM} P100 (Montañez-Izquierdo et al., 2012).

Apart from temperature, multiplicity of infectivity, and treatment time, other factors may influence efficacy of biocontrol treatments, notably the presence of organic matter such as the food matrix. A further parameter which must be considered when examining efficacy of treatment on surfaces is the surface architecture itself, which may range from a smooth rendered surface to a scored surface with associated crevices which may be colonized by bacteria and their biofilms. Chaitiemwong et al. (2014) considered both surface crevices and food matrices (diluted food residues of ham, salmon, endive, or milk) when measuring the efficacy of Listex^{TM} P100 treatment. Results suggested deeper crevice features on the surface decreased the treatment efficacy, with inactivation in the magnitude of > 3-log achieved on 0.2 mm crevices compared to the max 1.4-log CFU/mL observed in crevice depth of 5 mm. Of particular note was the difference seen when comparing the food matrix, with lower inactivation observed for milk and vegetable when compared with meat or fish. Ganegama Arachchi et al. (2013) mimicked conditions in fish processing and demonstrated the presence of fish protein led to a lower associated biofilm density compared to control stainless steel experiments when a fish protein matrix was added to the cultivation of *L. monocytogenes* biofilm on stainless steel. This highlights the complex role the food matrix may play in both biofilm formation and subsequent efficacy of bacteriophage treatment, demonstrating the need for further studies to understand the significance of food matrix on bacteriophage treatment efficacy.

Taken together, current literature detailing phage biocontrol studies directed at *L. monocytogenes,* such as those detailed above, shows differing success in their ability to decrease established biofilms. The often low reductions achieved demonstrate the challenges biofilms pose for not only bacteriophages but all control methods, but this is not to say that there is no place for phages as a potential biocontrol method. As with many disinfection regimes, additional interventions such as steps to loosen biofilm or remove organic matter can increase the success of phage treatments (Ganegama Arachchi et al., 2013). Further research considering multi-species biofilms and in-facility application will help determine the true potential of this biocontrol approach.

ENDOLYSINS

Endolysins (lysins) are hydrolytic enzymes required for bacteriophage dissemination from the host bacterial cell. They occur at the end of the lytic cycle to release the phage virions by breaking down peptidoglycan in the bacterial cell wall in what is termed lysis from within (Chan and Abedon, 2015; Schmelcher and Loessner, 2016). Researchers have harnessed lysins through protein expression production systems, generally in *E. coli*. Following purification of the lysin, it can be applied externally to the cell wall, thus not requiring phage infection, for biopreservation and biocontrol application (García et al., 2010). Lysins are grouped based upon the cell wall component they attack with the five main classes being N-acetylglucosaminidases, endo-β-N-acetlyglucosaminidases, lytic transglycosylases, endopeptidases, and N-acetylmuramoyl-L-alanine amidases (García et al., 2010; Schmelcher and Loessner, 2016). Lysins are highly specific with a narrow spectrum of activity making them host specific with some lysins only being active on the bacterial strain the phage was isolated from (Oliveira et al., 2012). In addition, they are fast acting and no development of resistance has been reported to date (Schmelcher and Loessner, 2016). Most

research has occurred on Gram-positive bacteria using the lysis from without approach as the peptidoglycan layer is exposed. Although limited, antimicrobial activity of lysins on Gram-negative bacteria has been reported when used in conjunction with EDTA, a membrane permeablizer (Chan and Abedon, 2015).

The antimicrobial activity of lysins has mostly focused on infection control of staphylococcal bacteria. Other applications that have been considered include use in agriculture to prevent plant disease by either intense application of cell lysates expressing a chosen lysin or development of transgenic plants by incorporation of the lysin gene into the plant genome (Düring et al., 1993; Kim et al., 2004); as a rapid detection and imaging method of pathogenic bacteria (Schmelcher et al., 2010; Bai et al., 2016); and transformation of listerial bacteriophage endolysin encoding genes into dairy starter cultures as a biopreservation method (Gaeng et al., 2000). Antilisterial lysins isolated to date have predominately focused on the control of planktonic cells *in vitro* with promising results although further validation is required (**Table 1**). Only a few antilisterial lysins have been assessed in food products and the food matrix and environment have been found to affect the antimicrobial activity (Oliveira et al., 2012).

To date there is only one lysin, PlyLM, which has been tested against *L. monocytogenes* biofilms after 100 % susceptibility on planktonic *L. monocytogenes* and *Listeria innocua* cells was achieved (Simmons et al., 2012). PlyLM reduced the monolayer biofilm to the same level as the application of lysozyme and proteinase K. When used in combination with proteinase K, or both proteinase K and lysozyme, synergistic effects were observed, and the biofilm was effectively digested. However, biofilms were only grown for 24 h at 37°C, and therefore the efficacy of these enzymes under other conditions merits further investigation, for example, performance at lower temperatures which are more reflective of those of most FPEs. More research has been undertaken on staphylococcal biofilms, predominantly monospecies biofilms, which have achieved reductions in biofilm mass. Of interest is their efficacy against persister cells. Persister cells are metabolically inactive subpopulations of cells, which are "super-resistant" to antimicrobial agents such as antibiotics (Brooun et al., 2000; Wood, 2017). Studies have shown these persister cells occur as a subpopulation of bacterial biofilms, and as such can present a significant obstacle to biofilm inactivation by antimicrobials (Brooun et al., 2000; Singh et al., 2009). Several studies have shown a promising role for lysins to inactivate persister cells in biofilms (Gutiérrez et al., 2014; Schuch et al., 2017). The success being reported against staphylococcal biofilms suggests that the potential lysins may have against biofilms in a food production context, particularly in targeting *Listeria* biofilms, which are a significant problem in FPEs. Another phage enzyme, extracellular polysaccharide depolymerase, has also be shown to degrade biofilm EPS; however, they are highly specific to the strains the phage infects (Chan and Abedon, 2015). A similar approach targeting *L. monocytogenes* in biofilms could also present an alternative control measure.

COMPETITIVE BACTERIAL SPECIES

Competitive exclusion is where one bacterial species competes with another species over resources and/or space in a habitat, successfully reducing the number of cells or excluding that species (Hibbing et al., 2010). This competitive exclusion can be the result of the production of antimicrobials such as bacteriocins, organic acids either acting directly against the species it is competing with or acting on the environment altering the pH, or alternatively physically outcompeting other bacterial species for nutrients and/or space and limiting normal survival or proliferation of those competitive species. This strategy is typically categorized into three components: competition, where planktonic cells of both species are co-cultured for a period of time; exclusion, where the antagonistic species are grown to a biofilm cell density prior to the addition of planktonic cells of the target species; or displacement, in which the target species are grown to biofilm cell density prior to addition of planktonic antagonists (Woo and Ahn, 2013; Pérez-Ibarreche et al., 2016). As biofilms protect microorganisms from chemical cleaners and disinfectants, the use of non-pathogenic microorganisms may assist sanitation approaches in controlling, preventing, or eradicating unwanted species like food borne pathogens.

Competitive exclusion studies typically pit planktonic cells of the antagonist species (i.e., the species which will exert a competitive exclusion effect) against planktonic cells of the target species in a competition assay, grown together for a period of time facilitating biofilm formation. Daneshvar Alavi and Truelstrup Hansen (2013) used a short incubation time of 72 h which resulted in a 1-log decrease in *L. monocytogenes* cell density after application of *Serratia proteamaculans*. A similar reduction was also reported by Fox et al. (2014) of *L. monocytogenes* biofilm cell density after 96 h when grown in co-culture with *Janthinobacterium lividum*. However, greater reductions have been reported when cells were incubated for longer periods with results around log 4.5 and 5.5 on stainless steel coupons and polytetrafluoroethylene, respectively (Pérez-Ibarreche et al., 2016). Zhao et al. (2004) also reported higher magnitude reductions of 7.8-log reduction over 28 days at 15°C by two bacterial isolates, *Lactococcus lactis* (*Lc. lactis*) and *Enterococcus durans*. In another experiment performed at 8°C for 28 days, four isolates, including the previous two isolates were also capable of reductions around 7-log units. However, the higher reductions reported by Zhao et al. (2004) and Pérez-Ibarreche et al. (2016) were produced by lactic acid bacteria (LAB) whose inhibitory activity has been studied extensively for many years, particularly as probiotics (Jeong and Frank, 1994).

The inhibitory effect of LAB was further explored by Guerrieri et al. (2009) and Gómez et al. (2016) as a preformed biofilm preventing *L. monocytogenes* biofilm formation as part of the exclusion strategy. Gómez et al. (2016) tested a variety of LAB strains and found reductions ranged from 4- to 7-log units over 24 and 48 h; however, by 72 h, *L. monocytogenes* growth had increased by almost half fold of the control indicating that these strains were only capable of exclusion

TABLE 1 | Antilisterial lysins reported in literature, key summary, and application.

Endolysin	Reported findings	Use	Reference
Ply118	Rapidly lysed all Listeria strains tested and against three Bacillus species.	BC, IC	Loessner et al., 1995
Ply500		BC, IC	
Ply511	Rapidly lysed all Listeria strains tested against.	BC, IC	
PlyP35	Determined optimal temperature, NaCl, pH, and various ions conditions.	BC, IC	Schmelcher et al., 2012
PlyP40	Lysed *L. monocytogenes* strain and *L. innocua*.	BC, IC	Loessner and Schmelcher, 2010
PlyP825	Inhibited all growth in *L. monocytogenes* strains used.	BC, IC	Grallert et al., 2012
PlyPSA	Determined crystalized structure	RMD	Korndörfer et al., 2006
PlyP100	Lysed all *L. monocytogenes*, Listeria strains in cheese, and a *Bacillus subtilus* strain tested against	BC, BP	Van Tassell et al., 2017
LysZ5	Lysed *L. monocytogenes*, *L. innocua*, and *Listeria welshimeri*; reduced *L. monocytogenes* numbers in soya milk.	BC, BP	Zhang et al., 2012
PlyLM	Lysed all *L. monocytogenes* and *L. innocua* strains tested against; digested *L. monocytogenes* biofilms when combine with a protease.	BC	Simmons et al., 2012

BC, biocontrol; IC, infection control; RMD, rapid multiplex detection; BP, biopreservation.

within the first 24–48 h. However, *Lc. lactis* 368 strain was able to completely exclude the growth of *L. monocytogenes* for the entire period, although it should be noted that all experiments were performed at a relatively elevated temperature and as such lower temperatures reflective of many FPEs require further consideration. In comparison, Guerrieri et al. (2009) showed the potential of LAB bacteria at refrigeration temperatures with a *Lactobacillus plantarum* (*Lb. plantarum)* strain capable of a 4-log reduction over a 10-day period. Mariani et al. (2011) used the native biofilm microflora of wooden cheese ripening shelves to achieve a 1- to 2-log reduction over a 12-day period, although this reduction was less than that observed in Guerrieri et al. (2009) and Gómez et al. (2016).

The third strategy displacement, as reviewed by Woo and Ahn (2013), demonstrated that the use of planktonic antagonist LAB strains as a post-treatment control method targeting *L. monocytogenes* was less effective compared to pre-treatment, although two strains (*Lactobacillus paracasei* and *Lactobacillus rhamnosus*) were capable of a 3-log reduction in *L. monocytogenes* biofilm cell density over 24 h when incubated at 37°C.

While most studies are performed in laboratories, Zhao et al. (2006, 2013) took the concept of competitive exclusion a step further and looked at its applicability in poultry processing facilities. In a fresh poultry facility, two LAB strains (*Lc. lactis* and *E. durans*) were added to two enzyme-based cleaners and applied as a foam to selected drains four times in the first week and then two times for the following 3 weeks. Sampling continued for 18 weeks after the last treatment. Most drains experienced significant reductions within the first week after only four applications and all drains maintained lower levels of *Listeria* throughout the sampling period (Zhao et al., 2006). Importantly, two drains reported significant reductions 16 weeks after treatments finished. Similar parameters were applied to the application of the same strains at a ready to eat poultry processing facility. By the end of the first week of application, *Listeria* was not detected in five of the six drains with all drains reporting negative results between weeks 8 and 13 (Zhao et al., 2013). It should also be noted that the strains utilized were known to either possess nisin or other forms of antimicrobials; however, it was not elucidated if the inhibition was the result of the production of antimicrobials.

There have been some encouraging results in the use of LAB against *L. monocytogenes* biofilm cells in laboratory-based experiments (**Table 2**); however, very few have been trialed in actual FPEs, apart from Zhao et al. (2006, 2013). The results from their two studies have shown promising results as an alternative control method utilizing *E. durans* and *Lc. lactis*; however, further longitudinal research surrounding the in-facility application is required. In addition, the application of other bacterial species identified in some of the studies mentioned above, for example, *J. lividum* and *S. proteamaculans*, warrants in-facility testing. However, it should be noted that the LAB strains utilized for in-facility application studies were isolated from the production environment indicating that specific strains may work best in the environment they were isolated from and these strains may vary depending on the food industry.

Houry et al. (2012) reported the use of bacterial species in a novel biocontrol approach. In the study, they identified a subpopulation of bacilli known as bacterial swimmers which were capable of creating transient pores within the biofilm structure. By pre-treating *Staphylococcus aureus* biofilms with bacterial swimmers, which also produced an anti-stapylococcal bactericide, they achieved a greater inactivation of *S. aureus* in biofilm by facilitating access of toxic substances in the environment into the biofilm.

BACTERIOCINS

An important component of the competitive survival strategy of bacteria is the production of antimicrobial products. One group of ribosomally synthesized antimicrobials are the heat stable peptides known as bacteriocins (Cotter et al., 2005; Gálvez et al., 2008; Winkelströter et al., 2015). It has been suggested that most bacteria produce at least one bacteriocin and LAB are known to be prolific producers (Cotter et al., 2005). Most

TABLE 2 | Bacterial species active against *L. monocytogenes* and purported mode of action.

Bacterial species	Mode of action	Studies
S. proteamaculans	Sánchez et al. (2010) identified a bacteriocin-like substance was produced at low temperatures capable of inhibiting *L. monocytogenes*. Inhibition was suggested to be the result of Jameson effect.	Sánchez et al., 2010; Daneshvar Alavi and Truelstrup Hansen, 2013
J. lividum	Specific strain utilized not tested for antimicrobial compounds. *J. lividium* are reported to have antibacterial compounds capable of inhibiting Gram-positive bacteria (O'Sullivan et al., 1990).	Fox et al., 2014
Lc. lactis	Neither of the studies by Zhao et al. tested for production of a bacteriocin; however, this species has previously be reported to produce nisin.	Zhao et al., 2004, 2006, 2013
E. durans	Neither of the studies tested for the bacteriocin; however, this species has previously be reported to produce enterocin.	Zhao et al., 2004, 2006, 2013
Lb. plantarum 396/1	Inhibition was attributed to production of an organic acid.	Guerrieri et al., 2009
Lb. paracasei	May be the result of competition for sites and resources. As a probiotic strain it may produce bacteriocin, organic acid or hydrogen peroxide.	Woo and Ahn, 2013
Lb. rhamnosus	May be the result of competition for sites and resources. As a probiotic strain, it may produce bacteriocin, organic acid or hydrogen peroxide. A previous study isolated an antilisterial bacteriocin from this species (Jeong and Moon, 2015).	Woo and Ahn, 2013
Lb. sakei	Bacteriocin producing strain.	Pérez-Ibarreche et al., 2016
LAB – Lc. lactis 368, *Lb. helveticus 354*, *Lb. casei 40*, and *W. viridescens 113*	Not identified as bacteriocin-producing strains. May be result of biosurfactants, or exclusion by trapping (killing cells embedded in biofilm).	Gómez et al., 2016
Native microbial flora of cheese ripening wooden shelves	Established biofilms on active cheese ripening wooden shelves were used. Inhibition may have been the result of competition for sites and nutrients.	Mariani et al., 2011

bacteriocins have a narrow spectrum of activity, that is, they are active against the same species that produces them but the producer is immune to them, while some have a broad spectrum of activity acting on members within the same genus as well as other genera and species (Cotter et al., 2005). The mode of activity varies depending on the particular class of bacteriocin and can include pore formation, or inhibition of key cellular processes such as peptidoglycan production, DNA replication, mRNA, or protein synthesis, to name a few (Cotter et al., 2005). There are two main groups: Class I (also known as lantibiotics), peptides that undergo post-translational changes, and Class II, which do not (Cotter et al., 2013). Among the most well-characterized and successful bacteriocins to date is nisin, a Class I bacteriocin from *Lc. lactis* which has been approved for use in food as a preservative/additive by the World Health Organization, European Union, and the United States Food and Drug Authority (Cotter et al., 2005). A great deal of research has gone into identifying more bacteriocins active against *L. monocytogenes* planktonic cells and biofilms, an important arena as nisin resistance is slowly being reported.

Most studies can be classified into two groups based upon how the bacteriocin is applied: either as whole bacterial cells known or suspected of bacteriocin production, or alternatively the bacteriocin extract itself, applied either as a crude or semi-purified product. Their utility against preformed *L. monocytogenes* biofilms of varying times has been the subject of numerous studies, with some reporting promising results. For example, Gómez et al. (2016) assessed *Lc. lactis*, *Lactobacillus sakei*, and *Lactobacillus curvatus*, all known to produce nisin Z, sakacine A, and sakacine P, respectively, against 48 h preformed biofilms. *Lb. sakei* and *Lb. curvatus* were capable of complete inactivation over 72 h whereas the two *Lc. lactis* strains provided a 6-log reduction by the end of the test period. Winkelströter et al. (2015), however, were unable to produce results of a similar magnitude when *L. monocytogenes* was co-cultured with *Lb. paraplantarum,* only achieving 2-log inactivation at 24 and 48 h before decreasing by 72 h. Guerrieri et al. (2009) took an alternative approach and reported that *Lb. plantarum* and *Enterococcus casseliflavus* were able to inactivate *L. monocytogenes* 7-day preformed biofilms by 3.9- and 3.7-logs over a 10 day-period. Importantly, the results could be associated with bacteriocin production, as no changes to the pH were observed.

Another technique is extracting the bacteriocin in the form of cell-free supernatant (CFS), as a crude bacteriocin fermentate or semi-purifying the product. The antimicrobial activity of CFS has shown mixed success in co-inoculation studies to prevent the formation of biofilms by *L. monocytogenes*, with Camargo et al. (2016) reporting significant reductions after 24 h, whereas Bolocan et al. (2017) only observed between 1.6- and 3.6-log CFU/cm^2 reduction after 72 h depending on the media used. In the latter study, however, the CFS extract which produced the highest reduction was from an isolate known to also produce an organic acid which was not

removed, and therefore this result may not be associated solely to the antimicrobial activity of the bacteriocin. When Camargo et al. (2016) applied the CFS to 24 h preformed biofilms for 2.5 h, they found biofilm formation continued in some isolates.

Other researchers have compared the two methods, bacterial cells and extracts again with varying results. García-Almendárez et al.'s (2008) analysis on 4-day preformed biofilms demonstrated a crude bacteriocin fermentate from *Lc. lactis* known to produce nisin A was capable of a 2.7-log reduction over 24 h. However, a greater reduction over 5-logs was achieved when the *Lc. lactis* was applied for 6 h, then rinsed, and placed in a desiccator for five days. Whereas, Winkelströter et al. (2011) co-inoculated *L. monocytogenes* with *Lb. sakei* or its CFS and found that any decreases observed in the first 24 h were diminished with time, as results at 48 h were comparable to the pure culture levels. A promising approach by Pérez-Ibarreche et al. (2016) involved the supplementation of *Lb. sakei* cells with a semi-purified bacteriocin for 6 h, which resulted in a twofold reduction in *L. monocytogenes* numbers on the stainless steel surface, or an additional 1-log reduction on polytetrafluoroethylene.

As mentioned previously, the bacteriocin nisin has been approved for commercial purposes and has paved the way as an alternative biocontrol method. Research into bacteriocins has been performed with comparable results to the other non-commercial bacteriocins discussed above. Minei et al. (2008) found that nisin was capable of inhibiting *L. monocytogenes* biofilm formation for 9 h on stainless steel coupons, and although cell growth did recommence after this time, a 3.5-log inactivation was still maintained by 48 h. On the other hand, Henriques and Fraqueza (2017) shortened the treatment time to 5 min and even at the highest concentration, no activity was recorded, although activity was defined as a $\geq$ 5-log decrease.

From the above, it is obvious that results vary significantly depending on if bacteriocin producing bacterial cells or the bacteriocin extracts is used. Results from bacteriocin extracts can be correlated to the antimicrobial action of the bacteriocin with greater certainty; however, additional analysis is required particularly when whole cells are used to help ensure that the measured inhibition is not the result of competitive exclusion or the production of other antimicrobials such as organic acids. The co-inoculation and preformed biofilm studies reflect the ability of the bacteriocin to either prevent the formation or affect the removal of established biofilms in the FPE; however, the length of time the biofilms are grown for prior to the bacteriocin being applied also affects the antimicrobial activity as mature biofilms may provide better resistance. Although several studies show that promising results most require additional analysis at temperatures and other environmental conditions mirroring the FPE to identify potential candidates suitable for further testing. With the potential resistance to nisin arising, the identification of other bacteriocins is essential. In addition, the application of synergistic antimicrobials to further combat the development of resistance should be considered.

PLANT-DERIVED ANTIMICROBIAL PRODUCTS – ESSENTIAL OILS

An alternative to the use of chemicals, microorganisms, or their derivatives is the use of plant-derived antimicrobial products such as essential oils (EOs). Herbs and spices are commonly known to exhibit antimicrobial activity and have been used by various cultures for flavoring, as a food preservative or for medicinal purposes. EOs play a key role in protecting plants from bacteria, fungi, viruses, insects, and animals (Perricone et al., 2015). Traditional distillation, cold press/expressing, solvent extractions, and enfleurage methods have been used to extract EOs from plant-derived materials; more recently, modern techniques including microwave or ultra sound assisted extraction, pressurized extractions, and super critical fluid extraction have been used to obtain EOs from a variety of plant sources (including roots, wood, bark, twigs, leaves, seeds, buds, flowers, and fruits). However, the constituents and compositions of EOs vary significantly from high concentrations to trace amounts based upon the plant part, plant age, and extraction method used, in turn influencing their antimicrobial activity (Lemberkovics et al., 2004; Reyes-Jurado et al., 2014; Perricone et al., 2015; Xia et al., 2017). Key molecules in EOs with the most effective antibacterial activity are typically from aldehyde and phenol chemical classes which include compounds such as cinnamaldehyde, carvacrol, eugenol, or thymol (Bakkali et al., 2008; Perricone et al., 2015). EOs are able to permeabilize the cell membrane resulting in the leakage of ions or other cell content, and may also disrupt key genetic functions and/or cellular components like proteins, polysaccharides, phospholipids, fatty acids, and essential enzymes due to the lipophilic nature of EOs (Bakkali et al., 2008; de Oliveira et al., 2010, 2012a; Perricone et al., 2015).

While there are thousands of EOs described, it is reported around 300 of these have generally recognized as safe approval and are used commercially for flavoring or fragrance; however, more detailed information is required for their use as a biocontrol agent (Burt, 2004; Reyes-Jurado et al., 2014). Most research surrounding the antimicrobial activity of EOs focuses on their effects on planktonic cells of food spoilage and pathogenic bacteria either in standard laboratory conditions or in their application on food items. This application on food as a biocide has major limitations as higher concentrations are required potentially interfering with the sensory attributes of the food (Burt, 2004; Chorianopoulos et al., 2008). In addition, some components of food items, mainly fats, proteins, carbohydrates, water, salt, antioxidants, pH, and other preservatives or additives used may impact upon the activity of the EOs (Perricone et al., 2015). Further research is required to understand the impact EOs have on bacterial pathogens and in particular their ability to prevent or eradicate biofilms in FPEs. Some research is occurring within this space; however, there is limited research against *L. monocytogenes* biofilms with a few studies looking at the extracted EOs, the active components of specific EOs, or altering the EO chemical composition.

de Oliveira et al. (2010) assessed the EOs from fresh citronella (*Cymbopogon nardus*) and lemongrass (*Cymbopogon citratus*) leaves applied alone or in combination; however, it was the Citronella EO which demonstrated the highest reductions against both the 3 and 240 h preformed biofilms with complete reduction after 60 min of application. Similar results reported in another study by de Oliveira et al. (2012b) found 2% (vol/vol) Chinese cinnamon extract (*Cinnamomum cassia*) was capable of reducing a 48 h preformed biofilm to below the detection limit (2.84-log CFU/cm^2) after 20 min; however, both of these studies applied the EOs at temperatures above 20°C.

Essential oils contain a mixture of major and minor molecules responsible for their antimicrobial activity with some of the major components being explored further. The active components of clove (eugenol) and spearmint (carvone) EOs were tested on a 6 h preformed *L. monocytogenes* biofilm but were found to increase biofilm mass by Leonard et al. (2010). Citral and nerol, in contrast, both major components from lemongrass (*C. citratus*) and *Lippia rehmannii* (nerol only), displayed a similar reduction as the positive control ciprofloxacin.

Additional microbial species can also impact upon the activity of the EO or active component. For example, Leonard et al.'s (2010) study as mentioned above was on *L. monocytogenes* monospecies biofilms and reported a mixture of results among the EO and the various active components tested, whereas de Oliveira et al. (2012a) looked at the activity of Chinese cinnamon and its active component, Cinnamaldehyde, on a mixed biofilm of *L. monocytogenes* and enteropathogenic *E. coli* on stainless steel coupons dipped in reconstituted whole milk. The EO and cinnamaldehyde were both capable of reducing the mixed biofilm to below the detection limit of 2.84-log CFU/cm^2 whereas the EO and active components only provided reductions just over 2-logs on the *L. monocytogenes* biofilm. Chorianopoulos et al. (2008) examined the EO and hydrosol (by-product of the steam distillation) of *Satureja thymbra* (Pink Savory) and showed similar results when grown in a mixed biofilm with a food borne pathogen (*L. monocytogenes* and *Salmonella enterica*) and a spoilage bacterium (*Pseudomonas putida*). It was noted that the optimized application time was 60 min and any increase in time provided no additional reduction. The impact other microbial players may have on the activity of EOs requires further exploration in order to gain insights into the various relationships at play.

A common problem for the use of EOs as a biocontrol method on food products is the associated impacts on taste at concentrations required for appropriate antimicrobial effect. A process to concentrate the EOs for application at a lower volume with the same potentially high antimicrobial activity may be required in the case of some EOs. Krogsgård Nielsen et al. (2017) looked at emulsifying and encapsulating isoeugenol oil to increase the antimicrobial effectiveness at a smaller volume with the addition of electrostatic forces to attract negatively charged bacteria to positively charged EOs. Although the concept of emulsification and encapsulation sounds promising, the minimal biofilm eradication concentrations (MBECs) for the coated and uncoated emulsified products were only half a log lower than the pure isoeugenol when tested in standard laboratory medium at three temperatures (6, 12, and 25°C) and no difference was observed in carrot juice. This observation requires further exploration as the reductions in the MBEC did not correlate to observations under confocal microscopy. Of note was the morphological changes observed in the mixed biofilms of *Pseudomonas fluorescens* and *S. aureus* from uniform layers to clusters of numerous cells, which requires further research to determine if there are any implications.

As mentioned previously, the use of EOs at concentrations to exhibit sufficient antimicrobial activity has the potential to impart undesirable flavor and when applied in an FPE may also result in an excessive sensorial impact. In addition, the interactions of EOs with components of the food matrix from food debris may also impact on the applicability of EOs in food environments. Only a few studies have investigated the application of EOs to disrupt or prevent the formation of biofilms. Further research on parameters specific to industry will allow a better decision on the application of EOs as an alternative or supplementary biocontrol method.

CONCLUDING REMARKS

While current sanitation processes are effective against planktonic cells, the potential for tolerant strains to increase due to interactions at subinhibitory levels and the potential reliance on them as antimicrobials, as the case in the health industry, is a cause for concern. The ability to eradicate established biofilms and prevent new biofilms from being formed is a challenging task which food production managers are charged with, as biofilms can present increased food safety risks. A useful tool in understanding the microbial community is metagenomics analysis of the FPE. By understanding the FPE microbiome, valuable information can be gained regarding persistence or transience of strains. This facilitates source tracking of persistent strains, can identify other microbial species that may provide either a positive or negative effect on the target strain, and can identify strains surviving the disinfection processes (Dass and Anandappa, 2017; Doyle et al., 2017). From this information, the appropriate biocontrol method can then be determined. There have been some significant advances in the development of biocontrol methods, particularly bacteriophages that have progressed to commercial products with the results of some studies validating their progression to commercialization. The use of competitive bacterial species has also showed some promising results with the concept of utilizing antagonist strains isolated from the production environment providing individualized treatment options. Bacteriocins and endolysins have also shown their ability to significantly reduce established biofilms; however, they typically require some form of purification process to achieve these results. The sensory implications of EOs at concentrations required to exert antimicrobial effects are a limiting factor

in their use as a sole biocontrol method, and therefore they may find more appropriate utility as a supplementary method targeting non-food contact surfaces. However, like all biocontrol methods, efficacy can be impacted by a variety of factors including temperature or time the control method was applied for, the use of one species or multiple species biofilms, biofilm growth method, or surface matrix composition. Standardization in the assessment of novel biocontrol methods against biofilms is required, in addition to assessment under conditions reflective of FPEs before appropriate comparisons can be made.

AUTHOR CONTRIBUTIONS

JG and EF conceived the study and drafted the manuscript. All authors corrected and approved the manuscript.

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